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GONADOTROPHINS IN RABBIT SERUM AFTER INTRAVENOUS INJECTION

By D. R. LAMOND*

From the Department of Veterinary Physiology, University of Sydney, Australia

(*Received* 12 *October* 1959)

There is good evidence that there is a rapid decline in activity of human chorionic gonadotrophin (HCG) in body fluids after parenteral administration (Zondek & Sulman, 1945). In general, there is a rapid initial decline in activity amounting to approximately 50% in the first hour and this is followed by a more gradual decrease, so that by 24 hr after subcutaneous injection approximately 10% remains. Pregnant mare serum gonadotrophin (PMS) behaves quite differently from HCG, inasmuch as 24 hr may elapse before a 50% reduction is obtained (Catchpole, Cole & Pearson, 1935), and activity may be demonstrated 5 days after intravenous injection.

At the time of these studies little was known of the time relationships involved in the action of gonadotrophins on the ovary. Recently, however, Emmens, Claringbold & Lamond (1957) demonstrated that irreversible changes take place in the mouse ovary within a few hours of gonadotrophin administration. PMS and HCG appear to be equally rapid in their action. Furthermore, they elicit similar joint action in the early production of uterine growth-promoting substances (Lamond & Claringbold, 1958).

The ovarian weight response, however, is not qualitatively similar (Lamond, 1958). Part of the difference in action may be related to differences in the rate of elimination of the two substances from the body, and in particular from the blood. It was decided to re-examine the rate of loss of gonadotrophin activity of the blood of rabbits in order to gain more precise information than was hitherto available.

METHODS

Plan of experiment. Two quantities of each of three gonadotrophic substances were injected intravenously into pairs of rabbits and the rabbits were bled at varying times after the injection. The doses of gonadotrophin (i.u.) were as follows:

	Low	High
HCG: Pregnyl (Organon Laboratories, Ltd)	300	1500
Antuitrin 'S' (Parke, Davis, Ltd)	300	1500
PMS: Gestyl (Organon Laboratories, Ltd)	600	3000

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In this laboratory 1 i.u. of either Pregnyl or Antuitrin 'S' gives a mouse uterine weight response equivalent to that given by 2 i.u. of Gestyl when blood serum is used as a vehicle (see Diczfalusy & Loraine, 1955, for details of augmentation of HCG by blood serum).

Experimental details. Gonadotrophin dissolved in isotonic saline was injected into the ear veins of twelve mature male rabbits (2·04–3·38 kg body weight, see Table 2). The injection volume was 2 ml. Each rabbit was bled 10 min, 1 hr, 6 hr and 36 hr after injection. The volume of blood taken at each bleeding was approximately 5 ml. After clotting, the blood was left during the night in the refrigerator. Each serum sample was decanted next morning and stored frozen in two vials.

Randomly bred immature female mice were employed in the bioassay of gonadotrophin (see Claringbold & Lamond, 1957, for details of the method). Each serum was diluted with untreated rabbit serum which had been stored frozen and which was thawed immediately before dilution. A total of 0·1 ml. of mixed serum was injected subcutaneously and the mice were killed 44–46 hr later, when uterine and body weights were recorded. Preliminary studies had provided information on the approximate titres expected in each serum. As a precaution, however, a single dilution of each of 24 samples, representing each treatment group, was administered to pairs of mice. This was sufficient to establish the approximate titres with reasonable accuracy. The sera were then assayed in three series extending over 3 weeks. In the first series the 10 min samples were assayed, in the second the 1 hr samples were assayed and in the third the 6 hr and 36 hr samples were assayed.

Each individual serum, representing one of the treatment combinations, was assayed by using five mice in each of two groups at a $\log_2$ dose interval. Except where the gonadotrophin concentration in the serum was minimal (see Results), the doses selected fell on the linear portion of the log. dose–log. uterine-weight-response line.

Standard preparation. Since not all sera could be assayed at one time, it was necessary to account for changes in sensitivity in the mice by the use of a standard preparation. The substance chosen was a commercial preparation of PMS (Serogan, British Drug Houses). 1000 i.u. was dissolved in 5 ml. of isotonic saline before commencement of the assays, and stored in the refrigerator. The potency does not alter during storage (Lamond, 1957). When gonadotrophin in any one batch of sera was being assayed, comparison was made with the two groups of standard sera prepared by administering 0·083 i.u. of PMS per mouse for one group and 0·25 i.u. per mouse for the other. For each group fifteen mice were used in the first series (10 min samples), twenty mice in the second series (1 hr samples) and thirty in the third series (6 hr and 36 hr samples).

RESULTS

The results to be considered are the responses to the two doses of standard on each of the 3 days plus the responses to the two doses of each serum for all bleedings at 10 min, 1 hr and 6 hr and the 3000 i.u. PMS sera at 36 hr. One point assays were carried out on the 600 i.u. PMS and 1500 i.u. HCG and two of the four 300 i.u. HCG sera collected at 36 hr.

The first step in the analysis of the results was to compute the regression of uterine weight on body weight for each of the three series of assays. The regressions were homogeneous ($\chi^2_{(2)} = 1{\cdot}36$, $0{\cdot}5 < P < 0{\cdot}7$) and a uniform value was used to correct all response totals for variation due to body weight.

All relevant figures were then submitted to an over-all analysis of variance, a summary of which is shown in Table 1. It is clear that the

slopes were homogeneous, and there was no interaction with either days or treatments. This established the validity of the assays and the average slope was used to estimate the titres of sera where only one point on the dose–response line was determined.

It can be seen from Table 1 that the average response to the standard preparation varied significantly from one day to another. The responses in the second week were greater than during the first and third weeks. Such time-to-time changes in sensitivity are well known (Claringbold & Lamond, 1957). This finding, however, precludes a simple comparison of relative potencies of sera assayed on different days and confirms the

TABLE 1. Analysis of variance of the uterine weight assays

Source of variation	D.F.	Mean square
Slope	1	68,211*
Assays		
Standard between days	2	1,708*
within days	10	79
Sera	37	2,402*
Slope × assays	49	101
Within-group error†	400	72·6

* $P < 0{\cdot}001$; † 5 mice per group

necessity for a standard preparation. The potency of each serum was therefore determined in terms of the number of international units of the standard PMS per millilitre. This was done in two stages. The first calculation was of that amount by which each serum had to be diluted in order to be approximately equivalent in action to 0·25 i.u. of the standard, which is the dose giving a uterine weight response corresponding to the point of inflexion of the log. dose–log. response curve. This was termed the median dilution factor (M) and was calculated by the general formula,

$$\log_3 M = \frac{m-a}{b} - C,$$

where
m = response to 0·25 i.u. PMS,
a = mean response to unknown,
b = slope, and
C = coefficient of dose.

Since M represents the amount by which 0·1 ml. serum has to be diluted to give a response equivalent to 0·25 i.u. PMS, the titre was calculated from the formula,

$$T = 2{\cdot}5M.$$

The results are presented in Table 2 and shown graphically in Fig. 1. The $\log_3$ median dilution factors for the HCG substances were submitted

to analysis of variance, as shown in Table 3. The analysis of the PMS data was carried out separately and is also shown in Table 3.

The HCG preparations gave similar results. With both levels of gonadotrophin there was a rapid initial decline in titre and this was followed by a period of very gradual decline. The size of the dose of gonadotrophin injected into the rabbits did not affect the rate of decline since the average slopes of the $\log_3$ median dilution–$\log_6$ time of bleeding (hours) curves were not significantly different one from the other.

There was an approximate decline of 50% in the amount of the PMS preparation detectable in the serum over the 36 hr period. The over-all rate of decline was again apparently independent of amount of gonadotrophin injected into the rabbit. An unusual feature of the responses is the significantly smaller titres at 1 hr compared to 6 hr.

TABLE 2. $\log_3$ median dilution factors (M) and titres (T), expressed as units of PMS standard per millilitre

Pregnyl

	1500 i.u.				300 i.u.			
	Rabbit 1 (2·62 kg)		Rabbit 2 (3·25 kg)		Rabbit 1 (3·13 kg)		Rabbit 2 (2·77 kg)	
Time of bleeding	$\log_3 M$	T	$\log_3 M$	T	$\log_3 M$	T	$\log_3 M$	T
10 min	2·5497	41·25	2·9923	66·75	0·6241	4·95	0·8898	6·65
1 hr	1·9868	22·15	1·7444	17·00	0·2828	3·40	0·4679	4·17
6 hr	0·9229	6·90	1·0177	7·65	−0·2662	1·87	−0·1713	2·07
36 hr	0·2007	3·12	0·2619	3·32	−1·7722	0·35	(−1·8000)	(0·3)*

Antuitrin 'S'

	1500 i.u.				300 i.u.			
	Rabbit 1 (3·30 kg)		Rabbit 2 (2·59 kg)		Rabbit 1 (2·70 kg)		Rabbit 2 (2·40 kg)	
Time of bleeding	$\log_3 M$	T	$\log_3 M$	T	$\log_3 M$	T	$\log_3 M$	T
10 min	2·1780	27·50	2·8810	59·25	1·0000	7·50	1·1194	8·55
1 hr	2·4048	35·00	2·1117	25·50	0·5955	4·80	0·6217	4·95
6 hr	0·9811	7·35	1·1402	8·75	−0·3634	1·67	−0·0224	2·45
36 hr	−0·4456	1·52	−0·2551	1·90	−1·4592	0·50	(−1·8000)	(0·3)*

Gestyl

	3000 i.u.				600 i.u.			
	Rabbit 1 (3·38 kg)		Rabbit 2 (2·78 kg)		Rabbit 1 (2·46 kg)		Rabbit 2 (3·20 kg)	
Time of bleeding	$\log_3 M$	T	$\log_3 M$	T	$\log_3 M$	T	$\log_3 M$	T
10 min	2·9615	64·50	3·2237	86·25	1·3364	10·85	1·8576	19·25
1 hr	2·1908	27·75	2·8582	57·75	1·1250	8·60	0·8536	6·37
6 hr	2·8187	55·25	3·1453	79·25	1·2617	10·00	1·1372	8·72
36 hr	2·2583	30·00	2·6770	47·00	0·7585	5·75	0·4116	3·92

Figures in brackets are live weights of rabbits.

* The response was minimal with two sera; the figures chosen are approximately the lowest titres which could be detected by the mouse uterine assay.

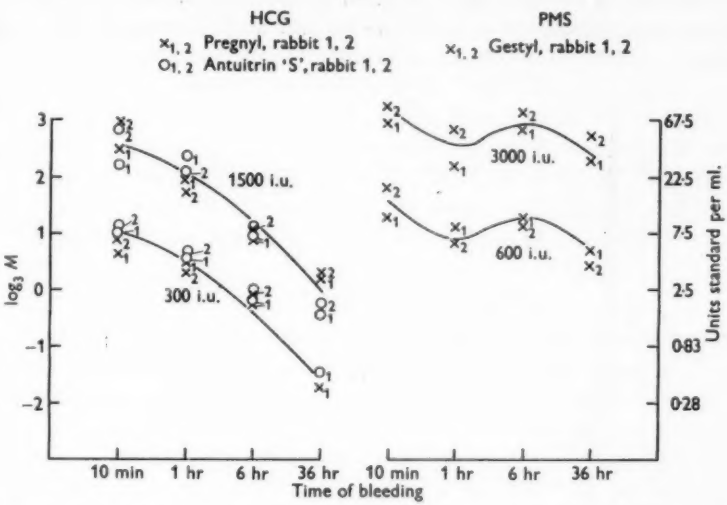

Fig. 1. Activity of gonadotrophins in serum at varying times after intravenous injection. The symbols represent the dilution of serum giving a mouse uterine response equivalent to 0·25 i.u. of a standard preparation of gonadotrophin. The curves were fitted by the least squares method.

TABLE 3. Analysis of variance of $\log_2$ median dilutions

Source of variation	D.F. HCG	D.F. PMS	Mean square HCG	Mean square PMS
Between times of bleeding				
Linear	1	1	31·4073‡	0·9002†
Quadratic	1	1	1·2361‡	0·0009
Cubic	1	1	0·0009	0·6624*
Between gonadotrophins				
Preparations (P)	1	—	0·0179	—
Levels (L)	1	1	19·1055‡	11·2089‡
$P \times L$	1	—	0·1401	—
Gonadotrophins × times	9	3	0·0979	0·0382
Remainder (error)	14§	8	0·0454	0·0788

* $0{\cdot}01 < P < 0{\cdot}05$; † $0{\cdot}001 < P < 0{\cdot}01$; ‡ $P < 0{\cdot}001$; § Degrees of freedom reduced by 2 owing to missing plots.

DISCUSSION

The rate of loss of activity of both HCG and PMS in the serum of rabbits was independent of dose over the range of doses studied. The general shape of the curves is shown in Fig. 2, in which the HCG curve is seen to be similar to that described by Zondek, Sulman & Sklow (1941).

The PMS curve is much flatter than that for HCG. An unusual feature of the results described in this paper, however, is the rapid decline in

concentration in the first hour followed by a significant rise. The level at 6 hr was approximately 70% of the 10 min level, compared with 50% at 1 hr.

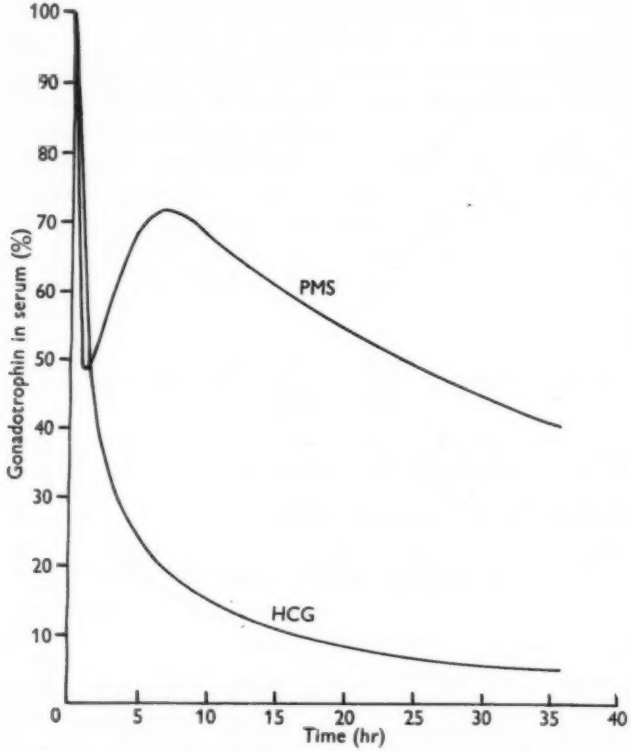

Fig. 2. Change in activity of placental gonadotrophins in rabbit serum after intravenous injection. The curves are constructed from the results described in Fig. 1.

TABLE 4. Augmentation of gonadotrophin by rabbit serum

Ratio of gonadotrophin levels observed 10 min after injection to expected gonadotrophin levels, calculated on the basis of dilution in plasma. The volume of plasma in the rabbit was assumed to be 40 ml./kg.

Gonadotrophin (i.u.)	Ratio, observed:expected titres	
	Rabbit 1	Rabbit 2
Pregnyl { 300	2·08	2·48
(HCG) { 1500	2·89	5·81
Antuitrin 'S' { 300	2·68	2·32
(HCG) { 1500	2·41	4·09
Gestyl { 600	1·79	4·11
(PMS) { 3000	2·91	3·20

In Table 4 are listed the ratios of concentration of gonadotrophin in serum observed at 10 min after injection to those expected in the plasma on the assumption of simple dilution in a volume of plasma corresponding to 40 ml./kg body weight (Darien Smith & Simmonds, 1954). It is apparent that both HCG and PMS as assayed by the uterine weight assay were augmented by the serum. An augmentation of approximately twofold was expected with HCG but not with PMS (see Methods). These results seem to indicate that there may be an active augmentation of PMS by rabbit serum *in vivo* which is not apparent when PMS is added to serum *in vitro*.

SUMMARY

1. The rate of decline of gonadotrophic activity of blood sera after the intravenous injection of placental gonadotrophins into mature rabbits was determined by using the mouse uterine weight bioassay for gonadotrophin.

2. The rate of loss of activity both for human chorionic gonadotrophin (HCG) and for pregnant mare serum gonadotrophin (PMS) was independent of the amount of gonadotrophin injected. The PMS curve, however, was much flatter than the HCG curve.

3. The potency of both substances was greater than that expected from simple dilution in serum.

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REFERENCES

Catchpole, H. R., Cole, H. H. & Pearson, P. B. (1935). Studies on the rate of disappearance and fate of mare gonadotropic hormone following intravenous injection. *Amer. J. Physiol.* **112**, 21–26.

Claringbold, P. J. & Lamond, D. R. (1957). Optimum conditions for biological assay of gonadotrophins. *J. Endocrin.* **16**, 86–97.

Darien Smith, I. & Simmonds, W. J. (1954). The changes in cardiac output, right atrial pressure and blood volume in haemorrhagic anaemias in unanaesthetised rabbits. *Aust. J. exp. Biol. med. Sci.* **32**, 241–252.

Diczfalusy, E. & Loraine, J. A. (1955). Sources of error in clinical bioassays of serum chorionic gonadotropin. *J. clin. Endocrin.* **15**, 424–434.

Emmens, C. W., Claringbold, P. J. & Lamond, D. R. (1957). Action of gonadotrophins on the ovary. *Nature, Lond.*, **180**, 38–39.

Lamond, D. R. (1957). Relative potency of some commercial gonadotrophic preparations. *Aust. vet. J.* **33**, 127–128.

Lamond, D. R. (1958). The action of different preparations of human chorionic gonadotrophin on the mouse ovary. *J. Endocrin.* **17**, 218–222.

Lamond, D. R. & Claringbold, P. J. (1958). Joint action of gonadotrophic substances. *J. Endocrin.* **16**, 298–303.

Zondek, B. & Sulman, F. (1945). The mechanism of action and metabolism of gonadotrophic hormones in the organism. *Vitam. & Horm.* **3**, 297–336.

Zondek, B., Sulman, F. & Sklow, J. (1941). Rate of disappearance of human chorionic gonadotrophin following parenteral administration in rats. *J. Endocrin.* **2**, 362–367.

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EFFECTS OF TEMPERATURE ON THE 5-HYDROXY-TRYPTAMINE (SEROTONIN) CONTENT OF TISSUES

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5-Hydroxytryptamine (5-HT) is known to be present in the mucosa of the gastro-intestinal tract, and in spleen, brain and platelets. These tissues are the main stores for 5-HT. Although the pharmacological actions of 5-HT have been investigated, its physiological function is not yet known. A number of compounds have been shown to release 5-HT from these tissues, but no positive conclusion can be derived from these experiments as to whether 5-HT alone is a mediator for the actions of these compounds (Paasonen & Karki, 1959). This paper records the results of experiments conducted to investigate whether submitting rats to different physical stimuli will vary the 5-HT content of spleen, gastro-intestinal tract and brain.

METHODS

Male albino rats weighing from 200 to 400 g were used, and were starved for 18 hr before the experiments. Some animals were exposed to heat by placing them under a row of electric light bulbs, the air temperature being 40–45° C; as a result, the rectal temperature rose from 36 to 39–42° C after half an hour. Other animals were exposed to cold by keeping them in a cold room at 1° C; this caused a fall in the rectal temperature from 36 to 30° C after half an hour. In some experiments lesions were made in the brain stem by means of a stereotaxic instrument with a current of 1 mA for 20 sec.

Electrical stimulation. Rats were placed in a Perspex cage with a wire netting floor which was divided in half by an insulating strip. The two halves were connected to the terminals of an inductorium. When the forelegs of the rats were on one half of the wire netting floor and the hind legs on the other half, the rats were stimulated by light electric shocks.

Pentobarbitone anaesthesia. Each rat was injected intra-peritoneally with 0·2 ml. of veterinary 'Nembutal' (12 mg pentobarbitone sodium; Abbott Laboratories).

Extraction of tissues. The rats were anaesthetized with ethyl chloride and bled to death. Tissues were minced and extracted with acetone (4 ml./g tissue) for 5–6 hr and again with two volumes 80% acetone during the night. The extracts were combined and evaporated to dryness *in vacuo* and the residues used for assay.

Assay of 5-hydroxytryptamine. 5-HT was assayed on the isolated rat colon (Dalgliesh, Toh & Work, 1953). The colon was suspended in a 20 ml. bath containing 0·2 μg atropine. For assay the amounts of tissue extracts added to the bath were equivalent to 5 mg stomach, 5 mg intestine, 10 mg spleen and 50 mg brain. Under these conditions the contractions of the rat colon produced by the extracts were completely abolished by bromlysergic acid diethylamide (BOL 148). If larger amounts of extracts were used, the assay for 5-HT was

no longer accurate, owing to the presence of other substances in the crude extracts which also caused the colon to contract. The contractions produced by these interfering substances were not abolished by BOL 148.

RESULTS

5-HT content of brain

The brain was dissected out and the cerebellum discarded. The cerebrum together with the brain stem including the medulla was extracted with acetone and assayed for 5-HT. In a group of six normal control rats the mean 5-HT content of brain was 0·38 μg/g tissue.

A group of six rats was exposed to heat for half an hour. Their mean brain 5-HT content was 0·20 μg/g tissue. In another group of six rats which had been exposed to cold for a similar period it was 0·24 μg/g tissue. The mean 5-HT content of the brains of these two groups of rats was significantly lower than that of the normal control group ($0{\cdot}001 < P < 0{\cdot}01$).

Another group of six rats was placed in a Perspex cage with a wire netting bottom through which light electrical shocks could be administered to the feet of the rats by means of an inductorium. This stimulation was carried out for 15 min. The mean 5-HT content of the brains of these rats was 0·45 μg/g tissue. Although this value was not significantly different from that of the control group, it was higher than the mean 5-HT content of the groups which had been exposed to heat and cold ($P < 0{\cdot}001$).

In a group of six rats bilateral electrolytic lesions were placed in the hypothalamus. In 3 days five of these rats had lost 20–50 g body weight; in one rat there was an increase of 8 g. At the end of this period they were killed and their brains extracted for measurement of 5-HT. A mean 5-HT value of 0·50 μg/g brain was found in these rats. This figure was not significantly different from the value of 0·38 μg/g brain of the control group ($P > 0{\cdot}1$), but was higher than the mean values of the groups exposed to heat and cold ($0{\cdot}001 < P < 0{\cdot}01$).

These results (see Table 1) show that the lower 5-HT content of the brain of rats exposed to extremes of temperature was unlikely to be due to the stressful conditions of heat and cold, because there was no loss in brain 5-HT in rats which had been subject to the stress of electrical shock. Hyperpyrexia has been shown to produce cellular changes in the hypothalamus (Brody & Robard, 1959) but in the group of rats which had electrolytic lesions placed in the hypothalamus there was no loss of brain 5-HT. It must therefore be supposed that when rats are exposed to extreme heat and cold a release of 5-HT occurs in the brain.

TABLE 1

5-HT content of brain of rats (μg 5-HT/g tissue)

	Control	Heat exposed	Cold exposed	Electric shocked	Hypothalamic lesions
	0·45	0·15	0·25	0·35	0·50
	0·44	0·15	0·25	0·60	0·70
	0·40	0·15	0·20	0·40	0·30
	0·40	0·15	0·20	0·40	0·65
	0·30	0·30	0·30	0·50	0·40
	0·30	0·30	0·25	0·45	0·45
Mean ± S.E.	0·38 ± 0·027	0·20 ± 0·032	0·24 ± 0·015	0·45 ± 0·037	0·50 ± 0·062
Significance of difference from control		$0·001 < P < 0·01$	$0·001 < P < 0·01$	$0·1 < P < 0·2$	$0·1 < P < 0·2$

Effect of pentobarbitone on 5-HT content of brain

A group of four rats was anaesthetized with pentobarbitone and exposed to heat for half an hour. At the end of this period their rectal temperatures had risen to 42–45° C. The mean 5-HT content of their brains was 0·37 μg/g tissue. This was significantly greater than the mean brain 5-HT content of unanaesthetized rats similarly exposed to heat, which was 0·20 μg/g tissue ($0·001 < P < 0·01$).

Another group of four rats was also anaesthetized with pentobarbitone and exposed to cold. After half an hour their rectal temperatures had fallen to 28° C. During the cold exposure the rats shivered. The mean 5-HT content of their brains was 0·31 μg/g tissue. Unanaesthetized rats also exposed to cold for the same period had a mean brain 5-HT value of 0·24 μg/g tissue ($0·01 < P < 0·02$). Pentobarbitone anaesthesia therefore inhibited the release of brain 5-HT in rats exposed to heat and cold.

Effect of 5-hydroxytryptophan (5-HTP) on 5-HT content of brain

Two rats were each injected intraperitoneally with 10 mg 5-HTP and killed after 1·5 hr. Their brain 5-HT content was 0·6 and 0·7 μg/g tissue. Two other rats were similarly injected with 5-HTP and after 1 hr they were exposed to heat for 0·5 hr. At the end of this period their brain 5-HT content was 0·5 and 0·6 μg/g tissue. No loss in brain 5-HT could be demonstrated under these conditions.

5-HT content of gastro-intestinal tract and spleen

The 5-HT content of the stomachs, small intestines and spleens of rats when the animals were subject to different physical stimuli was also studied. No significant difference was found in the 5-HT content of these organs between control rats, rats exposed to heat or cold, rats which were electrically shocked through the feet or rats which were killed 3 days after

the placing of hypothalamic lesions in them (Table 2). In the group of rats which had hypothalamic lesions one of the animals had ulcers in the stomach mucosa but its intestinal mucosa appeared normal. The 5-HT content of this stomach was 10 μg/g tissue and in the intestines it was 3·5 μg/g tissue.

TABLE 2

5-HT content of gastro-intestinal tract and spleen (μg 5-HT/g tissue)

	Stomach	Small intestine	Spleen
Control	5·0, 5·5, 5·5, 6·5 (5·6 ± 0·32)*	5·5, 7·0, 8·0. 5·5 (6·5 ± 0·61)	3·0, 2·8, 2·5, 2·5 (2·7 ± 0·12)
Heat exposed	6·0, 8·0, 5·0, 7·0 (6·5 ± 0·65)	4·5, 7·0, 1·5, 8·0 (5·2 ± 1·45)	2·0, 4·0, 2·0, 2·5 (2·6 ± 0·47)
Cold exposed	5·0, 5·0, 5·0, 5·5 (5·1 ± 0·13)	5·5, 4·0, 3·0, 3·5 (4·0 ± 0·35)	2·0, 2·5, 3·0, 5·0 (3·1 ± 0·66)
Electric shocked	5·0, 6·0, 7·0, 4·5 (5·6 ± 0·55)	5·0, 7·0, 3·0, 4·5 (5·1 ± 0·84)	3·0, 4·0, 3·5, 3·5 (3·5 ± 0·20)
Hypothalamic lesions	8·0, 10·0, 3·0, 5·0 3·5, 5·0 (5·8 ± 1·11)	10·0, 3·5, 3·0, 4·0 3·0, 5·0 (4·8 ± 1·11)	4·0, 5·0, 1·3, 1·8 3·0, 2·5 (2·9 ± 0·57)

* Figures within brackets give mean values ± S.E.

DISCUSSION

In the central nervous system 5-HT has been found to be concentrated in the brain stem, the hypothalamus containing the largest amount of 5-HT (Amin, Crawford & Gaddum, 1954; Paasonen & Vogt, 1956; Bogdanski, Weissbach & Udenfriend, 1957). It is virtually absent or present only in very small amounts in the cerebellum. It is because of this general finding that in these experiments the cerebellum has been excluded and 5-HT has been determined in the cerebrum and brain stem. Although it is recognized that 5-HT is widely distributed throughout the central nervous system, it is not known whether the substance is located in excitable neural tissue or in other types of cells in the C.N.S. Any attempt to interpret the behavioural reactions of an animal as being due to the appearance or disappearance of one particular substance in the C.N.S. is unjustified. Although the experimenter may discover a correlation between the reduction of one particular substance and the experimental technique, it is equally likely that the same experimental technique will bring about a similar reduction in the content of other substances which were not investigated. Thus, initial experiments with reserpine showed that administration of this drug brought about a reduction in the 5-HT content of brain. It was concluded that the central nervous effects of reserpine were therefore mediated by a loss of 5-HT and that 5-HT has an important part to play in C.N.S. function (Pletscher, Shore & Brodie, 1956). It was, however, soon discovered that reserpine also reduced the noradrenaline content of brain (Holzbauer & Vogt, 1956). No final conclusion can

therefore be made as to whether the action of the rauwolfia alkaloids is mediated by 5-HT or noradrenaline or both (Paasonen & Karki, 1959). It may even be possible that reserpine brings about a release of other substances in the C.N.S. besides 5-HT and noradrenaline.

Similarly the observation that the 5-HT content of brain in rats exposed to extremes of temperature is lower than in other rats must be interpreted with caution. A reduction in 5-HT content of brain may mean that its release is faster than its synthesis. If synthesis is as rapid as release then no reduction will be observed, even though release may have occurred. It is therefore interesting to note that rats which have been injected with 5-HTP, the precursor of 5-HT, do not show a reduction of brain 5-HT when exposed to heat. It is possible that, in the presence of a plentiful supply of its precursor 5-HTP, synthesis of 5-HT is rapid enough to keep pace with release, so that no reduction of 5-HT content can be demonstrated. These experiments do not indicate whether the loss of brain 5-HT is brought about by changes in brain temperature *per se* or whether the release is due to excessive stimulation of sensory temperature receptors. The injection of 5-HT into the internal carotid artery in dogs (Douglas & Toh, 1953) or intraventricularly in cats (Feldberg & Sherwood, 1954; Gaddum & Vogt, 1956) produces an increase in respiration. It is recognized that panting in lower animals is an attempt to regulate body temperature when they are exposed to heat. Does it mean then that the release of 5-HT in the C.N.S. is a physiological regulation to maintain a constant body temperature? The central action of 5-HT, however, is not limited to respiratory stimulation. It has been also observed that 5-HT releases adrenocorticotrophic hormone from the pituitary of the rat but does not do so if the rat has lesions in the hypothalamus (Smelik & de Wied, 1958). In experiments reported here, however, rats subject to the stress of electrical shock did not show a reduction of brain 5-HT.

The barbiturate anaesthetics are known to act on the hypothalamus (Masserman, 1937). It is therefore interesting that rats anaesthetized with pentobarbitone do not show a loss of brain 5-HT when exposed to heat or cold. However, no reduction in brain 5-HT content could be demonstrated in rats which have had lesions placed in the hypothalamus 3 days previously.

In spite of the variety of experimental procedures the 5-HT content of spleen and gastro-intestinal tract did not show any significant change. These experiments do not therefore throw any light on the physiological function of 5-HT in these tissues. Even the role of 5-HT in platelets, which are the richest source of 5-HT, remains obscure (Zucker, 1959).

SUMMARY

1. The 5-HT content of the brains of rats exposed to extreme heat or cold is lower than that of control rats, of rats subject to the stress of electrical shock or of rats with hypothalamic lesions.

2. No significant alteration in the 5-HT content of spleen, stomach, and small intestines has been observed in rats subject to these experimental procedures.

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REFERENCES

Amin, A. H., Crawford, T. B. B. & Gaddum, J. H. (1954). The distribution of substance P and 5-hydroxytryptamine in the central nervous system of the dog. *J. Physiol.* **126**, 596–618.

Bogdanski, D. F., Weissbach, H. & Udenfriend, S. (1957). The distribution of serotonin, 5-hydroxytryptophan decarboxylase and monoamine oxidase in brain. *J. Neurochem.* **1**, 272–278.

Brody, H. & Rodbard, S. (1959). Chromatolysis and vacuolation of specific diencephalic nuclei induced by acute pyrexia. *Amer. J. Physiol.* **196**, 33–35.

Dalgliesh, C. E.., Toh, C. C. & Work, T. S. (1953). Fractionation of the smooth muscle stimulants present in extracts of gastro-intestinal tract. Identification of 5-hydroxytryptamine and its distinction from substance P. *J. Physiol.* **120**, 298–310.

Douglas, W. W. & Toh, C. C. (1953). The respiratory stimulant action of 5-hydroxytryptamine (serotonin) in the dog. *J. Physiol.* **120**, 311–318.

Feldberg, W. & Sherwood, S. L. (1954). Injections of drugs into the lateral ventricle of the cat. *J. Physiol.* **123**, 148–167.

Gaddum, J. H. & Vogt, M. (1956). Some central actions of 5-hydroxytryptamine and various antagonists. *Brit. J. Pharmacol.* **11**, 175–179.

Holzbauer, M. & Vogt, M. (1956). Depression by reserpine of the nor-adrenaline concentration in the hypothalamus of the cat. *J. Neurochem.* **1**, 8–11.

Masserman, J. H. (1937). Effects of sodium amytal and other drugs on the reactivity of the hypothalamus of the cat. *Arch. Neurol. Psychiat., Chicago*, **37**, 617–628.

Paasonen, M. K. & Vogt, M. (1956). The effect of drugs on the amounts of substance P and 5-hydroxytryptamine in mammalian brain. *J. Physiol.* **131**, 617–626.

Paasonen, M. K. & Karki, N. T. (1959). Increase of 5-hydroxytryptamine in the rat brain by raunescine. *Brit. J. Pharmacol.* **14**, 164–167.

Pletscher, A., Shore, P. A. & Brodie, B. B. (1956). Serotonin as a mediator of reserpine action in brain. *J. Pharmacol.* **116**, 84–89.

Smelik, P. G. & de Wied, D. (1958). Corticotrophin releasing action of adrenaline, serotonin and pitressin. *Experientia*, **14**, 17.

Zucker, M. B. (1958). Serotonin (5-hydroxytryptamine): Hematologic aspects. In *Progress in Hematology*, **2**, 206–224, ed. Tocantins, L. M. New York: Grune and Stratton.

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THE RELEASE OF HISTAMINE AND FORMATION OF A SLOW-REACTING SUBSTANCE (SRS-A) DURING ANAPHYLACTIC SHOCK

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The release of histamine during anaphylaxis has been demonstrated in several species and various tissues, but it is well known that histamine alone cannot satisfactorily account for all the effects on smooth muscle observed during the anaphylactic reaction. The experiments presented in the present paper show that in addition to the release of histamine, the antigen–antibody reaction results in the formation of another smooth-muscle-stimulating substance which causes a slow and long-lasting contraction of the guinea-pig ileum, and which is resistant to anti-histamine drugs. This substance will be referred to as SRS-A.

The existence of a slow-reacting substance in the effluent collected during shock from the perfused lungs of the guinea-pig was recognized by Kellaway & Trethewie (1940). They were, however, unable to separate its effect upon the guinea-pig gut from that of histamine in the effluent, and their evidence for its presence was based on the observation that the active effluent caused a more prolonged contraction of the gut than did histamine alone. The use of anti-histamine drugs now makes it possible to study SRS-A separately.

METHODS

The experiments were performed on perfused lungs or minced tissue from sensitized animals.

Sensitization. Young albino guinea-pigs, usually male, of 200–250 g were given an intraperitoneal and a subcutaneous injection each of 100 mg dried egg albumin, as a 10% solution in saline, containing 0·5% phenol. The animals were killed with N_2O, or by dislocation of the neck, 3–5 weeks later, when they were always found to be strongly sensitized to the antigen.

Young male sandy-lop or albino rabbits were used. A primary antibody response was produced by two intramuscular injections of 25 mg of the antigen, in 1 ml. of adjuvant medium (Freund & McDermott, 1942) given 7 days apart. After 6 weeks, 5 or 6 graded doses of alum-precipitated antigen, rising from 2 to 10 mg, were given intravenously at intervals of 3 days. The animals were killed by dislocation of the neck, or bled out under pentobarbitone, 6–10 days after the last injection.

Rats of both sexes, of a hooded strain maintained at the National Institute for Medical Research, London, and weighing about 120 g, were sensitized similarly to rabbits but with

half doses (Lipton, Stone & Freund, 1956). They were killed 5–8 days after the last injection, by a blow on the head, after which the area of damage was at once isolated by passing a tight band round the neck.

Male rhesus monkeys, weighing about 2·5 kg, were sensitized by 50 mg antigen, in 2 ml. of Freund's adjuvant medium, given intramuscularly in two portions deep beneath each scapula, followed after 8 days by 80 mg of soluble antigen subcutaneously. Three weeks later the animals were bled out under pentobarbitone.

Tissue from a sensitized goat was kindly provided by Dr J. H. Humphrey. The course of sensitization was similar to that described for rabbits, and the animal was bled out under pentobarbitone 10 days after the last dose of antigen.

The lungs of a horse which had been used for the commercial production of anti-diphtheria serum were kindly provided by Dr A. C. White (Messrs Burroughs Wellcome, Beckenham). The animal had a high titre of circulating antibody, and was bled out under chloral hydrate anaesthesia.

On two occasions it was possible to obtain human lung removed from asthmatic subjects at operation. On one occasion, the tissue was immediately placed in a large volume of aerated Krebs–Henseleit (1932) solution at about 4° C. On the other, the tissue was kept wrapped in gauze at about 20° C for 1 hr before it was placed in aerated Tyrode solution at about 10° C. Both pieces of lung were obtained through the good offices of Dr A. W. Frankland of the Wright–Fleming Institute, who also provided details of the case histories and performed skin tests on the patients. Only those segments of the lobe which showed no gross evidence of disease were taken.

Lung perfusion. The intact lungs of the guinea-pig, rat, rhesus monkey and rabbit were perfused through the pulmonary artery via a flexible cannula which was passed through the right ventricle and was tied in well proximal to the bifurcation of the artery. The whole tissue was suspended by the trachea, inside a jacketed vessel which was maintained at 37° C and was lightly closed at the top. The bottom of the vessel was funnel-shaped, to collect the effluent solution. Perfusion fluid was supplied from a Marriotte bottle, and passed through a warming coil to raise its temperature to 37° C, immediately before it entered the cannula. Antigen solutions were warmed to 37° C, and injected into the perfusion fluid through a short rubber connexion between the warming coil and the cannula. In some experiments the left auricle was cannulated for the collection of the effluent, but as this gave no apparent advantage the fluid was usually allowed to flow out through an incision in the auricle. The lungs were usually air-perfused (Arunlakshana & Schild, 1950), but sometimes simply inflated through a cannula in the trachea. The rate of air perfusion was adjusted to maintain an initial pressure of 5–10 mm Hg, at the trachea; the air escaped from the alveoli through light scarifications made with a sharp triangular needle on the surface of the inflated lungs. Broncho-constriction was registered as an increased pressure difference across the lung. During the anaphylactic reaction broncho-constriction is intense and air flow becomes negligible. Air perfusion was usually stopped inmediately after injecting the antigen, or alternatively the pressure was limited to 40 mm Hg, in order to avoid interference with the flow of perfusion fluid through the blood vessels, and damage to the tissue proximal to the broncho-constriction, by over-distension with air. Unless otherwise stated, perfusion of the lungs of guinea-pigs with Tyrode solution began between 5 and 8 min after death, at a rate of about 10 ml./min. After 2 or 3 min the rate was reduced to 2 or 3 ml./min, the antigen being added to the inflow 15–20 min later. The lungs of other species were similarly flus ed free from blood, and then perfused at 1–2 ml./min/g tissue. In the case of the monkey and horse, where the lung could not be perfused within 10 min of death, the tissue was either left undisturbed in the animal for a period of not more than 10 min, or was removed with minimal handling and kept in oxygenated Tyrode or Krebs–Henseleit solution at about 10° C for not longer than 1 hr. Samples of perfusate were collected into test-tubes and immediately chilled in ice. In some experiments, the collecting tube was

surrounded by ice, but this did not seem to be necessary. The samples were then centrifuged, and if not immediately used were stored at −5 to −15° C.

Minced tissue. Sensitized guinea-pigs were killed by breaking the neck, and at once perfused by two routes, one cannula entering the pulmonary artery, and another entering the aorta; both auricles were cut open. Tyrode solution at 37° C, supplied at a pressure of 30–40 cm water, was used for perfusion, which continued for 10 min. Samples of various tissues were then removed, and after being lightly blotted and weighed, were placed in previously aerated Tyrode solution at 0–2° C. The samples weighed between 190 and 210 mg, except in the cases of aorta, vena cava and suprarenal cortex, where the available tissue weighed only 90–150 mg. The tissues were cut up with sharp scissors whilst still in this solution, to give particles of about 1 mm diameter, washed and incubated for 10 min at 37° C in 5 ml. Tyrode solution with oxygen bubbled through it. The Tyrode solution was then drained off and replaced by 2 ml. of fresh Tyrode solution at 37° C and incubated whilst being rocked and aerated as described by Mongar & Schild (1956). After 5 min the solution was removed, kept for assay of the spontaneous release of active substances, and at once replaced by 2 ml. of Tyrode solution containing antigen (ovalbumin) 50 μg/ml. and incubated similarly for a further 5 min, after which the solution was removed from the tissue and kept at 0° C for assay the same day.

Extraction of SRS-A from lung tissue. In some experiments SRS-A was extracted from lung tissue of the guinea-pig. Since SRS-A present in freeze-dried samples of perfusate was found to be readily soluble in 75 % aqueous ethanol, this solvent was used to make protein-free extracts of tissue. The lung was lightly blotted and weighed, then dropped into absolute ethanol chilled in ice, and cut into small pieces with sharp scissors. Absolute ethanol 3 ml./g tissue was used and later adjustments in volume were made with 75 % ethanol. The tissue was then ground up thoroughly with sand and allowed to stand at 22° C for 1 hr, after which the solution was filtered off and made up to volume through the filter. This extract was evaporated to dryness under reduced pressure, then 2 ml. distilled water and 10 ml. Tyrode solution were added before biological assay.

Biological assay. Assays for histamine and for SRS-A were routinely performed on a portion of the terminal ileum of guinea-pigs of 300–500 g. The tissue was suspended in oxygenated Tyrode solution at 37° C in an automatic apparatus, as described by Boura, Mongar & Schild (1954). A light frontal-writing lever system was used which exerted a pull of 0·5 g on the tissue, and gave a magnification of about × 5 on the kymograph.

Atropine 10^{-6} or 5×10^{-7} M was added to the Tyrode solution in the reservoir supplying the bath. Histamine acid phosphate was used to make the standard solutions, all values being expressed as histamine base. The assay for SRS-A was carried out in the presence of mepyramine 10^{-6} M. The values for SRS-A were expressed in arbitrary units, referring to 1/20 ml. of a standard sample of freeze-dried perfusate. This reference sample lost potency very slowly during storage; thus the unit is not constant throughout the whole series of experiments but permits valid comparisons within each group of experiments. A concentration of 1 unit/ml. was convenient for assays on the guinea-pig ileum.

Preparation of serum. In order to find out whether serum influences the output of histamine and SRS-A from perfused guinea-pig lung during the antigen–antibody reaction, guinea-pig serum was prepared as follows. Blood was taken by cardiac puncture, through a wide-bore needle coated with silicone, into a chilled, siliconed syringe. It was at once gently expelled into a siliconed centrifuge tube surrounded by ice, and then centrifuged at 2–6° C for 20 min at 1600 *g* or more. About two-thirds of the plasma was removed with a pipette, avoiding the plasma near the buffy coat and the uppermost portion. The plasma was allowed to clot at room temperature in a test-tube which contained a roughened glass rod. When the clot was firm (after about 15 min), the serum was expressed from the fibrin by gentle pressure between the rod and the wall of the tube. It was stored at 0° C for not more than 2 days. Serum so obtained was inactive on the guinea-pig's ileum when freshly

diluted with 3 volumes of Tyrode solution, but caused a slow contraction after being incubated with 10 volumes of Tyrode solution for 30 min.

Examination of guinea-pig blood for platelets. Some experiments were performed on the perfused lungs of guinea-pigs which had been depleted of their platelets by treatment with anti-platelet serum (Humphrey, 1955). In order to check this depletion, the blood was examined by the following procedure. 2 ml. of blood was withdrawn from the heart through a wide-bore needle coated with silicone, into a paraffin-coated syringe containing 2 ml. of 10% versene (di-sodium salt of ethylene-diaminetetraacetic acid) in normal saline, at pH 7·5, the whole having been chilled in ice. The blood and versene-saline were mixed at once, and after being transferred to a siliconed tube cooled in ice, were centrifuged at about 1600 *g* for 20 min at between 2 and 6° C. The entire buffy coat and 'carpet' of platelets was then cleanly removed with a pipette, and resuspended in cold versene-saline. A portion of this suspension was mixed with a solution containing formaldehyde and brilliant cresol blue, and examined in a Thoma haemocytometer cell.

RESULTS

Experiments on the perfused guinea-pig lung

The effluent collected from the perfused lung of a sensitized guinea-pig, after arterial injection of antigen, contained two substances which contracted the atropinized guinea-pig ileum. One substance was histamine, the other produced a much slower contraction and will be referred to as SRS-A. Figure 1 shows the assay of the effluent collected during the fifth minute after an injection of egg albumin to the perfused sensitized

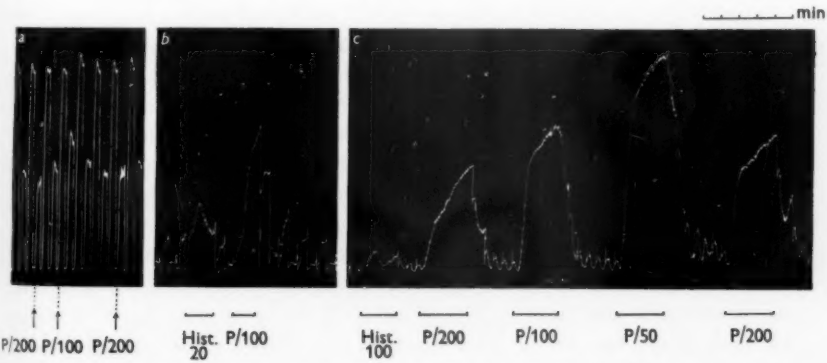

Fig. 1. The use of mepyramine to demonstrate the presence of both histamine and SRS-A in the perfusate (P) from shocked guinea-pig lung. The record shows the contractions of guinea-pig ileum suspended in Tyrode solution containing atropine 5×10^{-7} M. The concentrations of mepyramine used in (*b*) and (*c*) were present in the bath for 15 min beforehand, and at all times during the record. The kymograph began to move just before the dose of active substances was placed in the bath, and stopped when the bath was drained. *a*, Unlabelled contractions were produced by histamine 5 and 10 ng/ml. with a normal contact time (25 sec). *b*, In the presence of mepyramine 2×10^{-10} M, histamine 10 and 20 ng/ml. At 'Hist. 20' the dose was left in the bath for a longer time. *c*, In the presence of mepyramine 10^{-6} M. Movements recorded on base line are due to washing and refilling bath.

lung. This is a representative sample, containing only a trace of the antigen and avoiding most of the initial flood of histamine. Figure 1*a* shows that the histamine equivalent of the sample was approximately 1 μg/ml. When the test was repeated, in the presence of mepyramine 10^{-6} M (Fig. 1*c*), the perfusate sample caused a slow, well maintained contraction, whereas histamine alone in equivalent doses had no effect. This contraction must, therefore, be due to SRS-A, which is shown to have a rather flat dose–response slope, and to exert a prolonged effect so that the tissue becomes increasingly responsive to it. In Fig. 1*b* the effect of histamine 10 ng/ml. has been almost abolished by mepyramine 2×10^{-10} M, so as to show that the slower contraction caused by SRS-A adds on to that caused by histamine. This figure also shows that the effect of SRS-A is persistent, causing an enhanced response to histamine for several minutes following a dose of perfusate, even though the bath fluid was changed twice after each contraction. This persistence and summation of action is also seen in Fig. 1*a* and makes it probable that the histamine equivalent of the perfusate is slightly higher than its true histamine content, although at the dilutions used the histamine contraction has reached maximum before the SRS-A contraction has begun. The effect of SRS-A was not attenuated by mepyramine, since comparable contractions were produced in the presence of concentrations of 10^{-4} M and 10^{-7} M.

The presence of SRS-A in the perfusate collected during the first 2 or 3 min did not seriously interfere with the assay of histamine on the atropinized guinea-pig's gut. These samples contained upwards of 1 μg histamine/ml. and less than 50 units of SRS-A/μg histamine: in later samples the presence of a greater proportion of SRS-A tended to make the estimates of the histamine content too high. The magnitude of this interference was demonstrated when samples of perfusate were re-assayed after acid hydrolysis. The method employed (Code, 1937) destroys SRS-A but not histamine. From the results plotted in Fig. 2 it is clear that the histamine values of the first samples containing histamine 2–5 μg/ml. were practically the same whether assayed without treatment or after acid hydrolysis, but in all later samples the values obtained after acid treatment were lower. Sometimes these values were only 50% of those obtained before treatment, whereas acid hydrolysis of comparable solutions of histamine acid phosphate (0·2 μg/ml.) entailed a loss of 10–15% of the biological activity. The discrepancy of up to 40% must be attributed to interference by SRS-A.

Polypeptides with smooth-muscle-stimulating activity are known to be formed after plasma has been diluted (Schachter, 1956) or kept in contact with glass (Armstrong, Jepson, Keele & Stewart, 1957). Perfusate assayed within a few seconds of leaving the lung had the same SRS-A

activity as perfusate stored for several hours at 0 or 20° C, showing that SRS-A enters the perfusion fluid during its passage through the lung, and does not develop after collection. This finding excludes the possibility that enzymes which might be released from the lung during anaphylaxis, or might become activated as a result of the dilution of tissue fluids, might then form SRS-A from some substrate in the perfusate after collection, and points to the lung tissue as the source of SRS-A.

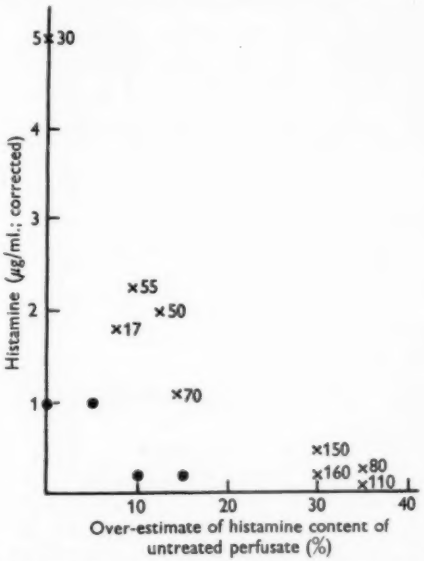

Fig. 2. The effect of SRS-A on the accuracy of the biological assay of histamine. Crosses refer to the percentage difference in the histamine assay attributable to the presence of SRS-A. The figures refer to the number of units of SRS-A, per microgram histamine, present in the various samples of perfusate. The circles ● represent the loss of biologically detectable histamine from histamine acid phosphate on acid hydrolysis. (Losses of this order have been allowed for in the estimation of the true histamine content of perfusate.)

Time course of release of histamine and the appearance of SRS-A in the effluent. In order to estimate the concentration of histamine and SRS-A in successive samples of perfusate collected from the lung after the injection of antigen, the samples were first assayed for histamine and the assay was then repeated for SRS-A in the presence of mepyramine 10^{-6} M. Owing to the dead space of the inflow cannula and the pulmonary artery, antigen injected during perfusion at the usual rate did not reach the capillaries of the lung until about 5 sec later. This was evident when dye was injected. Since histamine and SRS-A were present in the effluent collected during

the first 30 sec after the injection of antigen, both substances must have left the tissue within 25 sec of the arrival of the antigen. Figure 3 shows that the maximal rate of histamine release was reached at about 1 min, whereas the maximal release of SRS-A occurred between the second and third minute. The figure also illustrates that the output of SRS-A decreases more slowly than that of histamine, and that appreciable amounts of SRS-A are still present 1 hr after the reaction with antigen, when histamine is no longer detectable in the effluent.

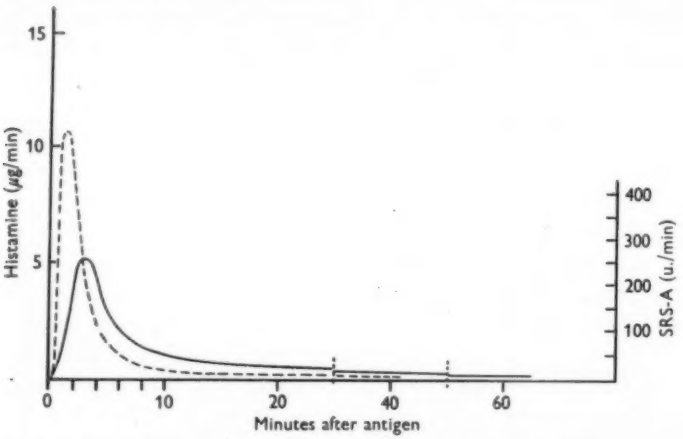

Fig. 3. Time course of the effluence of histamine and SRS-A from guinea-pig lung after the arrival of antigen: compiled from 15 experiments, each lung weighing 2–3 g. Histamine – – –, SRS-A ——. SRS-A 1 u./ml. usually caused a contraction of the guinea-pig's ileum equal to between 5 and 7 ng histamine/ml. In terms of activity in this tissue, the graph magnifies the effect of SRS-A about × 3.

The lung tissue as the source of SRS-A. The results of the following three series of experiments show that the SRS-A in the effluent is not derived from constituents of blood which might be present in the perfused lung at the time the antigen is given.

(*a*) The lungs from matched pairs of litter-mate guinea-pigs were perfused for different times or at different rates, so that one was more thoroughly freed from blood than the other. The amounts of SRS-A and histamine which were released from the two lungs by antigen, under otherwise similar conditions, were compared with each other. The presence of an appreciable amount of blood in the lung when the antigen was administered always decreased the amounts of both substances released; the yield of SRS-A was reduced rather more than that of histamine. It is thus unlikely that SRS-A was derived from the blood. When the lungs were perfused for 30 min or longer, or when the rate of perfusion exceeded

5 ml./min for 15 min or longer, the yields of both SRS-A and histamine were decreased, the decrease of SRS-A being greater than that of histamine. Optimum yields of both substances were obtained when the lungs were perfused at a rate of 2–4 ml./min for 15–20 min before the addition of the antigen. These conditions were therefore adopted in subsequent experiments.

(*b*) The addition of serum to the perfusion fluid did not affect the output of either SRS-A or histamine during anaphylactic shock. This was first shown when the lungs were perfused both before and after the addition of antigen, with Tyrode solution containing 5 or 10 % freshly diluted serum,

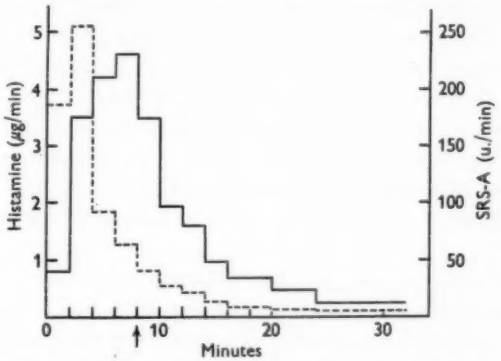

Fig. 4. The effect of adding guinea-pig serum at the arrow, to the perfusion during anaphylaxis produced by a steady level of antigen in the perfusing fluid.

and the total yields of histamine and SRS-A during 15 min compared with the yields from lungs perfused with Tyrode solution. When Ungar (1956) produced results showing that the histamine yield was increased when serum was added during the continuous administration of a low concentration of antigen, experiments based on this procedure were performed. After preliminary perfusion with Tyrode solution for 15 min, antigen in a concentration of 5 μg/ml. was added to the perfusion fluid. In the following 8 min the output of SRS-A reached its maximum. Perfusion was then continued with Tyrode solution containing 10 % of fresh serum from normal guinea-pigs, as well as the antigen. Figure 4 shows that in these experiments the output of SRS-A did not increase as a result of adding serum, but continued its normal decline.

(*c*) Substances which stimulate smooth muscle are present in platelets. To exclude the possibility that SRS-A might be derived from platelets adherent to the capillaries of perfused lungs, guinea-pigs were depleted of their plateletes by an intraperitoneal injection of 0·2 ml. of anti-platelet serum, 2 or 3 days before the lungs were used. Blood obtained by cardiac

puncture from treated animals contained 1 platelet or less/5 leucocytes, compared with more than 50 platelets/5 leucocytes in the untreated animals. Since the haematocrit was also considerably reduced, the loss of platelets per millilitre of blood was probably even higher. In spite of this great reduction in platelets, the addition of antigen to the perfusion fluid led to a substantial output of SRS-A. This was only 50–60 % of that obtained from the untreated controls, but this reduction cannot be related to the reduction in platelets. The reduction may be due to the damage which anti-platelet serum causes either directly or indirectly to capillaries throughout the lung, as was made manifest by the presence of haemorrhagic patches. These patches are probably perfused incompletely or not at all, and are therefore not subjected to the full effect of the antigen.

(*d*) In routine perfusions the heart is left attached to the lungs during perfusion and shock, and might contribute to the yields of histamine and SRS-A. When the heart alone was perfused via the coronary arteries, the average yield in three experiments was 2·5 μg histamine and 75 units SRS-A per gram of tissue. Only a fraction of this yield would be expected during lung perfusion in which the antigen makes only superficial contact with the heart.

Formation of SRS-A. The antigen–antibody reaction releases histamine from the stores of tissue histamine, but the SRS-A which appears in the effluent of the perfused lung is actually formed in the lung tissues. This is evident from experiments in which extracts of shocked and unshocked lung were tested on the mepyramine-treated guinea-pig ileum. For this purpose the right and left lungs were both perfused, but the antigen was added

TABLE 1. The histamine and SRS-A content of sensitized guinea-pig lung, and its effluent perfusate, before and after anaphylactic shock. Mean of six experiments

	Histamine (μg/g lung)		SRS-A equivalent (u./g lung)	
	Tissue	Perfusate	Tissue	Perfusate
Before antigen	17·5	0	350	0
After antigen	8·2	7·0	800	290

to only one side. Both the lungs were then extracted by grinding the tissue in ethanol, to give a final concentration of 10 ml. of 75 % alcohol/g tissue. All the extracts caused the mepyramine-treated gut to contract, but those from the shocked tissue were always more active than those from the corresponding unshocked half of the lung. In addition, the perfusate from the shocked lung contained SRS-A, whereas that from the control lung did not. The activity from the control lung was probably not due to SRS-A but to potassium and/or other normal constituents of the tissue.

The results, given in Table 1, show that SRS-A is formed within the

perfused lung by the antigen–antibody reaction, that some remains in the tissue and can be extracted from it. In contrast, the appearance of histamine in the effluent from the shocked lung is associated with a diminution of the histamine content of the tissue.

Experiments on the lungs of other species

Rabbit. Although the blood is generally considered to be the shock organ for anaphylaxis in the rabbit, the injection of antigen at the arterial inflow released both histamine and SRS-A from the perfused lungs of sensitized animals. The amounts of histamine released were relatively small, and varied in different experiments; they bore some relationship to the levels of circulating antibody, and were therefore presumed to reflect varying degrees of sensitization. Purified diphtheria toxoid, bovine serum albumin, crystalline ovalbumin, and crude egg albumin were all effective antigens. The mean yield of histamine from four strongly sensitized animals was 0·7 μg/g lung, and the corresponding yield of SRS-A was 46 units/g. The time course of histamine release differs from that observed in guinea-pig lung. The output does not reach its maximum rate until 2 or 3 min after the antigen. By the second minute the output of SRS-A is already high and remains so for upwards of 5 min, reaching a maximum 3–4 min after the injection of antigen. In one experiment SRS-A was present in the effluent 1 min before any histamine could be detected.

Rhesus monkey. The lungs of two monkeys were perfused. In each, the injection of antigen at the arterial inflow caused the appearance of both histamine and SRS-A in the effluent from the shocked lungs. The results are presented in Table 2. The amounts of histamine released were 2 and

TABLE 2. The histamine and SRS-A content of the perfusate from the isolated lungs of Rhesus monkeys during anaphylactic shock

	Experiment 1		Experiment 2	
Perfusion period (min)	Histamine (μg/min)	SRS-A (u./min)	Histamine (μg/min)	SRS-A (u./min)
Control	0	< 1	0	0
0–3	2·6	4	7·0	5
4–10	3·3	85	5·7	185
11–20	1·0	50	1·2	90
21–30	0·2	20	0·6	80
Total yield per gram lung in 30 min	2 μg	70 u.	5 μg	350 u.

The tissue was challenged with 30 mg antigen at time 0. A further 10 mg was given at the 7th minute in Expt. 1, and at the 20th minute in Expt. 2.

5 μg respectively, per gram fresh weight of lung. This is lower than the yield obtained from guinea-pig lung but represents a considerable total yield, since the lungs weigh about 20 g. The time course of histamine release

in the two experiments resembled that of rabbit. The amounts of SRS-A and histamine in the perfusate were in about the same proportion as from the guinea-pig lung. For instance, a release of 5 μg of histamine per gram would also have been associated in the guinea-pig lung with a release of SRS-A of the order of 300 units/g.

Man. On the two occasions when it was possible to perfuse segments of a lobe of lung obtained during operation, the allergens responsible for the asthma had been identified clinically. Both patients had been treated with antibiotics for tuberculosis, and although the disease was no longer active, lobectomy was performed because large cavities remained in the

TABLE 3. The histamine and SRS-A content of the perfusate from human asthmatic lung, when challenged with specific allergens

Perfusion period (min)	Histamine (μg/min)	SRS-A (u./min)
Before antigen	0	0
(→ Birch pollen)		
0–1	0·5	0
2–4	30	870
5–10	9	750
11–28	5	520
29–31	2	280
(→ Birch pollen)		
32	2	480
33–35	2	320
36–41	1·6	240
42–47	0·9	200
(→ Timothy pollen)		
48	1	200
49–51	1·5	340
52–75	0·6	165
76–78	0·3	130
Total yield	285 μg	27,000 u.

Perfusion of a 200 g segment from the middle left lobe of a woman allergic to birch tree and timothy grass pollens. 50 mg soluble allergens in 0·5 ml. saline, injected into inflow at time 0, and at the start of the 32nd and 48th minutes, as indicated.

lobe. Some segments of the removed lobe appeared to be healthy in both instances: these were dissected out and perfused with Krebs–Henseleit solution. When, after a preliminary period of perfusion, the allergens to which the patients had been sensitive were added to the perfusion fluid as it entered the lung, histamine and SRS-A appeared in the effluent. Table 3 gives the results of one of the experiments in detail. The segment was perfused 1½ hr after the lobe had been removed. During the whole of this time the tissue was stored in oxygenated Krebs–Henseleit solution at 4–10° C. A few minutes after perfusion was started the effluent became clear of blood, and the whole segment appeared to be uniformly perfused. The effluent collected before the addition of the allergen caused no contraction of the guinea-pig's ileum, but after the allergen (birch pollen) the

effluent contained considerable amounts of both histamine and SRS-A. The histamine was first detected 1 min after the allergen, but SRS-A could not be detected with certainty at that time, since the sample contained pollen solution which interfered with the assay. The sample collected during the second, third and fourth minutes contained 1·2 μg histamine and 75 units of SRS-A/ml., corresponding to an output of 30 μg/min and 900 units/min during this period. In subsequent samples the concentration of both substances decreased. When a second injection of birch pollen was given half an hour later, the output of both substances increased only slightly. The patient had also been sensitive to the pollen of timothy grass, and when a solution of this allergen was injected, a further small additional release of histamine and SRS-A occurred. The total amount of histamine released during the half hour following challenge with birch pollen was about 245 μg, and a further yield of 40 μg was obtained in the next 47 min, during which the two further doses of allergens were injected. The corresponding figures for SRS-A, expressed in arbitrary units, were 17,300 and 10,000. Since the segment of lung weighed approximately 200 g, the total release of histamine corresponded to 1·4 μg/g of tissue, and that of SRS-A to 135 units/g.

A small amount of tissue from the same lobe was used to test the reaction to the two allergens by the diffusion method (Mongar & Schild, 1956). When particles of chopped tissue were suspended in oxygenated Tyrode solution containing the allergen, both histamine and SRS-A were released (W. E. Brocklehurst & J. L. Mongar, unpublished).

Two of the allergens to which the second specimen of lung was sensitive were grass pollen and cat scurf. The perfusion of this lung specimen was incomplete and the tissue contained clotted blood, since storage after the lobectomy had been unsatisfactory. Grass pollen was tested first; it caused the release of both histamine and SRS-A. The subsequent injection of cat scurf led to a renewed output of histamine and SRS-A in the effluent, showing that the grass pollen had not desensitized the tissue against cat scurf. From a part of this specimen which was not used for perfusion bronchioles were dissected and chains of rings set up as isolated tissues, in Krebs–Henseleit solution. The addition of specific allergens to the bath caused contraction of the smooth muscle of the bronchioles, and both histamine and SRS-A were found in the bath fluid, when tested on the guinea-pig ileum.

Rat. The lungs of rats sensitized to crystalline ovalbumin or to human serum albumin were perfused. An injection at the arterial inflow of 10 mg of the antigen did not cause the appearance in the venous effluent of either histamine or SRS-A. This was not due to inadequate sensitization of the animals, since at the time of the experiment the blood contained between

1 and 6 mg of antibodies/ml., and intradermal injections of the antigen regularly produced local anaphylactic reactions in animals of the same group. Furthermore, the final intravenous dose of antigen given in the course of sensitization had in all cases produced prostration characteristic of anaphylactic shock. In a few experiments in which the liver or hind quarters of sensitized rats were similarly perfused, the addition of the antigen likewise failed to cause the appearance of histamine or SRS-A in the venous effluent.

Goat. The lungs of one goat, sensitized to rabbit serum albumin, were perfused after the animal had been bled out under pentobarbitone anaesthesia. No histamine or SRS-A appeared in the venous effluent after the addition to the perfusion fluid of 10 μg of the antigen/ml. At the time of the experiment the blood contained 5 mg circulating antibodies/ml., and mild anaphylaxis had been observed during the course of sensitization.

Horse. Two separate segments of the lungs from a hyper-immunized horse were perfused free from blood shortly after death. In both perfusions the injection of 2 ml of the antigen solution (a formolized culture of *Corynebacterium diphtheriae*) at the arterial inflow failed to cause the appearance of histamine or SRS-A in the venous effluent.

Experiments on minced tissues of the guinea-pig

The diffusion technique of Mongar & Schild (1956) was used to show that, in various tissues of the guinea-pig, the antigen–antibody reaction leads to the appearance of SRS-A in the Tyrode solution in which the tissue particles are suspended. The results are given in Table 4. The experimental procedure differed slightly from that of Mongar & Schild in that the tissues were perfused *in situ* to remove blood before being minced. This was necessary in order to prevent undue interference with the assay of SRS-A by plasma kinins and other smooth-muscle-contracting substances derived from the blood. The histamine released into the bathing fluid was also assayed, and the results are included in the Table.

The control values were obtained by incubation of the washed tissue fragments for 5 min in Tyrode solution without the addition of antigen. During this period some histamine was released into the bathing fluid from most tissues; the same result had been obtained by Mongar & Schild. With the exception of the lung and submaxillary gland, no substance resembling SRS-A was found in this bathing fluid, and in both these cases the amount was very small. After the Tyrode solution was removed from the tissue, the fragments were resuspended in Tyrode solution containing the antigen (ovalbumin, 50 μg/ml.), and again incubated for 5 min. With the exception of striated muscle fibres, liver and cerebral hemisphere, all tissues released histamine into the bathing fluid. The largest amounts of

histamine were released from the lung tissue, then in descending order, from vena cava, aorta, ileum, trachea and suprarenals. The histamine released from the sample of diaphragm did not originate from the muscle, because when muscle freed from pleura and peritoneum was minced and incubated with the antigen no histamine was released.

The quantities of histamine and of SRS-A appeared to be related in a few tissues, but this was not general. By far the highest amounts of both substances were obtained from the lung tissue. The vena cava, which released about 14% of the amount of histamine released by the lung,

TABLE 4. The amounts of histamine and SRS-A obtained by diffusion from various tissues of a sensitized guinea-pig. All tissues were perfused to remove blood before being cut into uniform fragments

Tissue	No. of expts.	Before antigen		Antigen present	
		Histamine (ng/g)	SRS-A (u./g)	Histamine (ng/g)	SRS-A (u./g)
Lung	7	200	< 10	7000	300
Trachea	5	30	0	500	0–25*
Ileum	5	60	0	600	0
Ileum without mucosa	2	100	0	500	0
Uterus	3	20	0	200	20
Uterus without endometrium	1	30	0	100	10
Aorta	7	60	0	800	40
Great veins	3	150	0	1000	40
Diaphragm	2	30	0	300	20
Diaphragm muscle only	2	0	0	0	0
Striped muscle (sartorius)	1	0	0	0	0
Skin	3	50	0	70	0
Liver	3	< 10	0	< 10	0
Spleen	6	0	0	200	0–5
Pancreas	4	< 10	0	250	10
Suprarenal	2	100†	0	500†	?5†
Suprarenal cortex	1	100	0	100	0
Submaxillary gland	2	10	<2	80	8
Cerebral hemisphere	1	0	0	0	0

* Variable. † Inhibitor substance (?adrenaline) present.

yielded about 13% of the SRS-A found in lung samples; figures for aorta were comparable. On the other hand, the bath fluid of ileum and trachea usually contained no detectable SRS-A, although relatively large amounts of histamine were released. A number of other tissues, which included salivary gland, pancreas and uterus, released relatively little histamine, yet the bath fluid always contained measurable amounts of SRS-A. The bath fluid from those tissues which released no histamine (liver, brain, striated muscle) did not contain SRS-A. Skin released only small amounts of histamine, and no SRS-A could be detected.

The bath fluid from some shocked tissues caused the mepyramine-treated gut to contract in a manner not characteristic of SRS-A. The type of contractions produced suggested that the active substance released from

skin may have been potassium, and that from the ileum might have been 5-hydroxytryptamine. The amounts were small. Samples from the suprarenals contained inhibitory substances, probably sympathomimetic amines, and produced contractions which resembled those due to SRS-A to which a trace of adrenaline had been added.

The absence of SRS-A in the bath fluid from some minced tissues incubated with antigen is not absolute proof that anaphylaxis does not normally lead to the formation of SRS-A in these tissues, because the method is less suitable for the detection of SRS-A than the method of perfusing whole organs. This was shown for the ileum. In three experiments the entire ileum from a sensitized guinea-pig was perfused through its artery, and the venous effluent was collected, precautions being taken not to collect fluid coming from the intestinal lumen. The effluent collected after the addition of antigen to the perfusion fluid contained readily detectable amounts of SRS-A, as well as histamine, but no 5-HT was found.

TABLE 5. The amounts of histamine and SRS-A obtained from guinea-pig ileum during anaphylaxis *in vitro*, by perfusion or diffusion

	Time (min)	Histamine (ng/g)	SRS-A (u./g)
Perfusion	0–10	50	3
	0–10	140	2
	0–5	250	6
	6–15	10	5
Diffusion	0–10	160	$< \frac{1}{2}$
	0–10	150	$< \frac{1}{2}$
	0–5	640	< 1
	0–5	600	$?\frac{1}{2}$

Antigen 10^{-4} g/ml. present continuously from time 0.

DISCUSSION

The present experiments on perfused and minced tissues of sensitized animals show that the antigen–antibody reaction leads not only to the release of histamine but also to the formation of a slow-reacting smooth-muscle-stimulating substance. Release of histamine and appearance of SRS-A, however, do not run parallel in all tissues.

Although the presence of a slow-reacting substance in the perfusate from sensitized lungs of guinea-pigs had previously been recognized by Kellaway & Trethewie (1940), they were unable to separate its effect from that of histamine, since at that time anti-histamine drugs were not available. In the present experiments mepyramine has been used to abolish the effect of histamine and reveals that of SRS-A.

SRS-A appeared as early as histamine in the effluent from the perfused lungs, although the maximum rate of output occurred about $1\frac{1}{2}$ min later

than the maximum output of histamine. This is in contrast to the conclusion reached by Kellaway & Trethewie, who stated that the slow-reacting substance was present only in later fractions of the effluent. Samples of effluent collected shortly after the antigen contain large amounts of histamine in comparison with the amounts of SRS-A, so that when they are tested on the guinea-pig's ileum, without the use of an antihistamine drug, it is necessary to dilute them very greatly, and the effect of SRS-A then becomes relatively insignificant. With the weakly sensitized animals used by Kellaway & Trethewie this would be particularly likely, and offers a reasonable explanation for their failure to detect SRS-A in early samples.

The amount of SRS-A formed during the anaphylactic reaction varies greatly with the kind of tissue, and is probably determined by the availability of the necessary substrate and the amount of enzyme activated during the reaction. The largest amounts were formed in lung tissue, and in tissue from arteries and veins, hence vascular tissue is a particularly good source.

In the guinea-pig the lung was found to be the major source of SRS-A; it is therefore the organ upon which experiments regarding the mode of formation can best be performed. The amount of SRS-A produced by the lungs is probably greater than that produced by all the rest of the body, and the local concentration produced in this tissue during anaphylaxis will exceed that reached anywhere else.

In the study of SRS-A the use of perfused organs has many advantages. Not only is the yield of SRS-A greater than that from minced tissue, but the resulting active solution is relatively free from protein, salts and other constituents of the tissue. This greatly increases the reliability of biological tests. In addition, the reaction takes place in tissue which has suffered no damage, has remained at or near 37° C, and can be aerated adequately during the perfusion; anaphylaxis therefore occurs under conditions resembling those which exist *in vivo*, with the sole exception that blood is absent.

The title SRS-A refers only to a principle possessing characteristic biological activity, and does not imply that this is necessarily a single substance. Nevertheless, this is probably so, since the biological activity is located in a single band after being submitted to electrophoresis (Charlwood & Gordon, 1958). SRS-A differs in its pharmacological properties from bradykinin, 5-hydroxytryptamine, substance P, and many other substances (Brocklehurst, 1953) and appears to be a principle not previously studied.

In the absence of more specific tests the identification as SRS-A of the biologically active substance released from tissues other than guinea-pig lung and human lung has been based on pharmacological criteria such as

the characteristic prolonged contraction and slow relaxation, the persisting stimulation made apparent by a following dose of histamine, the entire absence of tachyphylaxis, and the relation of dose to response when tested on the guinea-pig ileum.

The finding that SRS-A is formed in the course of the antigen–antibody reaction strongly suggests an enzymic mechanism. According to Mongar & Schild (1957) the release of histamine is also the result of an enzymic process. However, it is not likely that the enzymes are identical, because SRS-A formation is prolonged, whereas the release of histamine is 'explosive'. The release of histamine occurs suddenly and declines quickly, even when only part of the releasable histamine is freed; the enzyme responsible for this must therefore act only for a very brief period, whereas that which forms SRS-A remains active for some time.

In all species in which the antigen–antibody reaction in the lung resulted in release of histamine it also led to the formation of SRS-A, but there was no quantitative correlation between the two. The degree of sensitization affected the two phenomena to a different degree. Weakly sensitized guinea-pig tissue usually produced little SRS-A, and moderate yields of histamine (5–10% of tissue content), whereas strongly sensitized tissue yielded up to 5 times as much histamine, and often 20 times as much SRS-A. Further, the release of the same amount of histamine was accompanied by the formation of larger amounts of SRS-A in the two specimens of human lung than was usual in the lung of the guinea-pig.

According to Ungar (1956) the presence of serum enhances the release of histamine by antigen. This finding was not confirmed. The guinea-pigs used in the present experiments were more strongly sensitized than those used by Ungar, and particular care was taken to ensure that the serum used was not contaminated by products from the blood cells, and also that the proteolytic activity which develops as a result of clotting remained minimal. These modifications might be the reason for the discrepancy between the results; it is likely that the release of histamine during a low-level anaphylactic reaction is augmented by the addition of traces of proteolytic enzymes.

Rocha e Silva (1950) found that the perfused liver of the sensitized dog released only traces of histamine, when challenged by the antigen, unless the perfusion fluid consisted of whole blood without an anticoagulant. He attributed the release of histamine to the break-down of aggregates of platelets and leucocytes trapped in the capillaries. The role of blood would appear to vary greatly between species, since in the present experiments on the perfused lung of man, monkey and guinea-pig substantial yields of histamine and SRS-A were obtained from tissue which was virtually free from blood. In some experiments with guinea-pig lungs, blood was

deliberately left in the tissue, or the platelets were destroyed previously by an antiserum, but this had no commensurate influence on the output of histamine or of SRS-A. Therefore, at least as far as the guinea-pig is concerned, blood residues and platelets appear to play no essential part in the release of histamine, nor can they be considered as the source of SRS-A.

The present results are incompatible with the view of Rocha e Silva, Bier & Aronson (1951) that anaphylatoxin contributes significantly to the release of histamine in the antigen–antibody reaction. This view was based on their finding that anaphylatoxin released between 14·3 and 32·6 μg histamine from the perfused lungs of a guinea-pig, whereas Bartosch, Feldberg & Nagel (1932) had obtained a release of only 0·5–4 μg, and Daly, Peat & Schild (1935) a release of 0·17–12 μg, from perfused lungs during anaphylaxis. However, the lungs from strongly sensitized guinea-pigs used in the present experiments regularly yielded 6–12 μg histamine per gram of tissue, with occasional yields of 15 μg/g representing 40 μg or more per lung, in response to the antigen, without the participation of blood elements, and therefore without anaphylatoxin formation. Moreover, in these experiments histamine release began in less than 30 sec, and reached a maximum rate about 1 min after addition of the antigen, which is comparable with the latency observed in anaphylactic shock in the intact guinea-pig. This suggests that *in vivo*, as well as *in vitro*, the direct effect of antigen on the sensitized tissue is primarily responsible for the release of histamine. A mechanism based on anaphylatoxin formation would appear to require a longer time, because it would be necessary for blood proteases to be activated, forming anaphylatoxin, and for this to leave the blood stream before releasing histamine in the tissues.

The occurrence of SRS-A following the antigen–antibody reaction is not dependent upon the kind of antigen used; ovalbumin, bovine and human serum albumin, diphtheria toxoid, pollen extract, cat and horse scurf, and pneumococcus polysaccharide (SSS III) all released histamine and caused the appearance of SRS-A. The fact that Schild, Hawkins, Mongar & Herxheimer (1951) did not detect any substance other than histamine in the bath fluid in which human asthmatic tissue had been shown to contract to pollen and other antigens does not conflict with this conclusion, because their experiments were designed primarily to prove that histamine was released. The methods they used to assay the bath fluid would not be expected to reveal the presence of SRS-A. In the present experiments solutions which had bathed human bronchial rings during anaphylactic contraction were found to contain SRS-A when tested at concentrations higher than those suitable for histamine assay.

The release of histamine from rabbit lung has not been described

previously. In the present experiments substantial amounts were only released when the tissue was highly sensitized, and even then the release did not result in bronchomotor or vasomotor responses of the lung. Thus, the underlying biochemical mechanism appears to be the same in the rabbit as in the guinea-pig, but the smooth muscle of the rabbit's lung is less sensitive to histamine, and the tissue as a whole reacts less vigorously to the antigen. The finding that neither histamine nor SRS-A appeared in the effluent from perfused lungs of sensitized rats, shows that either the perfusion conditions which are suitable for the guinea-pig and rabbit are not satisfactory for the rat, or that there is some fundamental difference such as the ability of the lung tissue to fix antibody. The few experiments with tissue from the horse and goat suggest that in these species also the lung may not be involved in anaphylaxis, or that blood may be essential to the reaction.

The proven existence of a second smooth-muscle-stimulating substance in anaphylaxis, particularly in lung, does not detract from the importance of histamine in anaphylaxis, but offers an explanation for some smooth-muscle reactions which cannot readily be attributed to histamine.

SUMMARY

1. When antigen is injected at the arterial inflow of the perfused lung of a sensitized guinea-pig, the effluent contains histamine and a slow-reacting smooth-muscle-stimulating substance (SRS-A).

2. SRS-A was found to be resistant to mepyramine, and could therefore be assayed on the mepyramine-treated guinea-pig ileum.

3. The time course of the output of SRS-A from the perfused lung of sensitized guinea-pigs, after the addition of antigen, is slower in onset and more prolonged than that of histamine.

4. SRS-A originates from the lung tissue; platelets and other constituents of blood are not the source of it, and are not necessary for its occurrence.

5. Whereas histamine is released from pre-existing stores in the tissue, SRS-A is formed as a result of the antigen–antibody reaction in the lung.

6. SRS-A was present in the effluent, after the antigen had been added to the perfused lungs of sensitized rabbits and monkeys, and of asthmatic human patients.

7. No SRS-A or histamine was detected in the effluent from perfused lungs of rats, on the addition of antigen, although by other criteria the animals were sensitized.

8. When formation of SRS-A was studied in particles of tissue from various organs of sensitized guinea-pigs, it was found to occur chiefly in

lung and vascular tissue. Some formation occurred in particles of salivary gland, spleen, pancreas and uterus, but little or none was detected in particles of ileum, trachea and skin.

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REFERENCES

ARMSTRONG, D., JEPSON, J. B., KEELE, C. A. & STEWART, J. W. (1957). Pain-producing substances in human inflammatory exudates and plasma. *J. Physiol.* **135**, 350–370.

ARUNLAKSHANA, O. & SCHILD, H. O. (1950). The air-perfused bronchial tree. *J. Physiol.* **111**, 48*P*.

BARTOSCH, R., FELDBERG, W. & NAGEL, E. (1932). Das Freiwerden eines histaminähnlichen Stoffes bei der Anaphylaxie des Meerschweinchens. *Pflüg. Arch. ges. Physiol.* **230**, 129–153.

BOURA, A., MONGAR, J. L. & SCHILD, H. O. (1954). Improved automatic apparatus for pharmacological assays on isolated preparations. *Brit. J. Pharmacol.* **9**, 24–30.

BROCKLEHURST, W. E. (1953). Occurrence of an unidentified substance during anaphylactic shock in cavy lung. *J. Physiol.* **120**, 16*P*.

CHARLWOOD, P. A. & GORDON, A. H. (1958). Electrophoresis in a density gradient. *Biochem. J.* **70**, 433–438.

CODE, C. F. (1937). The quantitative estimation of histamine in the blood. *J. Physiol.* **89**, 257–268.

DALY, I. DE B., PEAT, S. & SCHILD, H. O. (1935). The release of a histamine-like substance from the lungs of guinea-pigs during anaphylactic shock. *Quart. J. exp. Physiol.* **25**, 33–59.

FREUND, J. & MCDERMOTT, K. (1942). Sensitization to horse serum by means of adjuvants. *Proc. Soc. exp. Biol., N.Y.*, **49**, 548–553.

HUMPHREY, J. H. (1955). The mechanism of Arthus reactions. II. The role of polymorphonuclear leucocytes and platelets in reversed passive reactions in the guinea-pig. *Brit. J. exp. Path.* **36**, 283–289.

KELLAWAY, C. H. & TRETHEWIE, E. R. (1940). The liberation of a slow-reacting smooth-muscle-stimulating substance in anaphylaxis. *Quart. J. exp. Physiol.* **30**, 121–145.

KREBS, H. A. & HENSELEIT, K. (1932). Untersuchungen über die Harnstoffbildung im Tierkorper. *Hoppe-Seyl Z.* **210**, 33–66.

LIPTON, M. M., STONE, S. H. & FREUND, J. (1956). Systemic and local anaphylaxis in the albino rat. *J. Immunol.* **77**, 453–461.

MONGAR, J. L. & SCHILD, H. O. (1956). Effect of antigen and organic bases on intracellular histamine in guinea-pig lung. *J. Physiol.* **131**, 207–219.

MONGAR, J. L. & SCHILD, H. O. (1957). Effect of temperature on the anaphylactic reaction. *J. Physiol.* **135**, 320–338.

ROCHA E SILVA, M. (1950). The role played by leucocytes and platelets in anaphylactic and peptone shock. *Ann. N.Y. Acad. Sci.* **50**, 1045–1067.

ROCHA E SILVA, M., BIER, O. & ARONSON, M. (1951). Histamine release by anaphylatoxin. *Nature, Lond.*, **268**, 465–466.

SCHACHTER, M. (1956). A delayed slow contracting effect of serum and plasma due to the release of a substance resembling kallidin and bradykinin. *Brit. J. Pharmacol.* **11**, 111–118.

SCHILD, H. O., HAWKINS, D. F., MONGAR, J. L. & HERXHEIMER, H. (1951). Reactions of isolated human asthmatic lung and bronchial tissue, to a specific antigen. *Lancet*, **261**, 376–382.

UNGAR, G. (1956). Mechanism of histamine release. *Ciba Foundation Symposium on Histamine*, pp. 431–449. London: Churchill.

J. Physiol. (1960), **151**, *pp.* 436–457
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AN INVESTIGATION INTO THE POSSIBLE EXISTENCE OF POLYNEURONAL INNERVATION OF INDIVIDUAL SKELETAL MUSCLE FIBRES IN CERTAIN HIND-LIMB MUSCLES OF THE CAT

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The 'motor unit' is classically considered to be an 'individual motor nerve-fibre together with the bunch of muscle fibres it activates' (Sherrington, 1930), and to be functionally separate from all other motor units. In the frog, however, such a simple arrangement is not found for the slow muscle fibres (Kuffler & Vaughan Williams, 1953). Instead, each muscle fibre is commonly supplied by more than one nerve fibre, a state of affairs which is termed polyneuronal innervation. A single fast muscle fibre of the frog may also have more than one end-plate upon it (Katz & Kuffler, 1941), and though these different end-plates may be supplied by branches of a single axon (Iwasaki, 1957), there is evidence that in some cases they are supplied by different axons (Cattell, 1928; Hunt & Kuffler, 1954).

In mammalian skeletal muscle histological methods have been widely applied, but have failed as yet to produce a final answer to the question whether or not polyneuronal innervation occurs normally. The difficulties are great, for it needs to be shown not only that individual muscle fibres have more than one end-plate, but also that these are derived from different axons rather than from the branches of a single axon. The subject has been reviewed repeatedly (Fulton, 1926; Hinsey, 1934; Denny-Brown & Pennybacker, 1938; Tiegs, 1953), and it has usually been concluded that if polyneuronal innervation occurs, then it is not on a large scale. Recently, in spite of a careful search, Feindel, Hinshaw & Weddell (1952) could find no anatomical evidence of polyneuronal innervation in various muscles of the rabbit. When in teased muscle preparations they occasionally found two end-plates upon a single muscle fibre they were always able to show that they were both supplied by the same axon. On the other hand, there is some evidence suggesting that polyneuronal innervation (hyperneurotization) may occur under certain abnormal conditions when regeneration of damaged nerve fibres is taking place (Hoffman, 1951*a*, *b*).

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Experimental work on mammalian muscle has mainly employed the method of recording the contraction of the muscle on stimulating two different nerves or motor roots supplying it. If the sum of the tensions produced by individual stimulation of each nerve is greater than the tension produced by the simultaneous stimulation of the two nerves, then polyneuronal innervation of some muscle fibres is suggested, for any musle fibres supplied by both nerves cannot contract more strongly when both nerves are stimulated together than they do when each nerve is stimulated alone. The early experiments performed along these lines gave variable results (Lederer & Lemberger, 1907; Agduhr, 1916; Quednau, 1926), and in any case can probably not now be considered conclusive, as certain possible sources of error may have been overlooked. Stevens & Karrer (1929), in careful experiments, found no tension deficit on stimulating the two nerves supplying the 'lateral head of gastrocnemius'. It would appear, however, that one of these nerves was that to the plantaris muscle (see Fig. 2, Jefferson, 1954), and an overlap of innervation might not be expected for such widely separated muscle fibres. More recently Jarcho, Eyzaguirre, Berman & Lilienthal (1952) studied the electromyogram of the rat's gracilis muscle before and after cutting intramuscular nerve branches. They concluded that occasionally there might be two widely separated end-plates upon a single muscle fibre, but their experiments did not permit them to decide whether these were innervated by the same or by different axons.

In contrast to most other evidence Hunt & Kuffler (1954) have recently claimed to have shown that polyneuronal innervation is common in the cat. Their most direct evidence was obtained by recording from single muscle fibres with a micro-electrode; but this was done only in the cricothyroid muscle, which, being innervated by cranial nerves, may not be typical of all somatic muscles. On limb muscles they performed only the classical experiment of stimulating two ventral roots, or two subdivisions of a single ventral root, and recording the twitch contraction of the muscle (soleus or flexor digitorum longus). Any tension deficit found on simultaneous stimulation of the two roots was expressed as a percentage of the tension produced by stimulation of the smaller of the two roots. This was taken as a measure of the percentage of the muscle fibres innervated by the smaller root which were also supplied by the larger root, and by this means they frequently found an 'overlap as great as 60–90 %'. There are, however, various other reasons which can be postulated for such tension deficits, and these do not all seem to have been excluded by Hunt & Kuffler's experiments. We have therefore repeated their experiments on the limb muscles and have also performed experiments of several additional types. In all these experiments the massed response of many muscle fibres

has been recorded, and single units have not been studied. In view of various complicating factors no one of the experiments alone could be considered conclusive, but taken together they have failed to produce any definite evidence for the existence of polyneuronal innervation. It therefore seems improbable that polyneuronal innervation occurs on a significant scale in the muscles studied, though it should be emphasized that its existence on a small scale has not been excluded. A preliminary communication has already been published (Brown & Matthews, 1959).

METHODS

Preparation. The experiments were performed on twenty cats weighing 1·5–4·7 kg, which were anaesthetized with pentobarbitone sodium (Nembutal; Abbott Laboratories) given intraperitoneally. The muscles studied were soleus (thirteen cats), flexor digitorum longus (seven cats) and flexor hallucis longus (seven cats). They were activated by stimulating an appropriate ventral root or portion of ventral root (L7 and S1 for soleus; L6 and L7 for the two other muscles). The other muscles of the leg were immobilized by wide-spread nerve section, leaving only the muscles studied innervated. (The following nerves were cut: femoral nerve, superficial and deep branches of the obturator nerve, hamstring nerve, lateral popliteal nerve, and all branches of the medial popliteal nerve except those supplying the muscle studied.) The nerve to soleus was dissected free from the lateral head of gastrocnemius through which it runs. Soleus was studied most intensively and these experiments were separate from those on the other two muscles, which were then denervated. Flexor digitorum and flexor hallucis were studied in the same preparations as each other, and both remained innervated throughout the experiment. Their contractions were, however, recorded at different times, and they were separated from each other by dissection of the distal parts of their bellies.

The nerve roots were exposed by lumbar laminectomy. The L6, L7 and S1 roots were cut close to the spinal cord and the dorsal roots of the same segments were ablated. This provided a 'nerve-muscle' preparation *in situ*, for the muscle studied was thereby denervated and its responses could not have been affected by any spinal reflex. On stimulation of the roots some hip or tail muscles contracted also, but section of the nerve to the muscle studied (at the end of the experiment) showed that this was not transmitted to the myograph. The exposed roots were covered in warm paraffin, which had been previously equilibrated with 95% O_2 + 5% CO_2. The muscle was left covered by skin, with its tendon protruding from a wound at the bottom of the leg. The pool in the leg and the laminectomy wound were kept warm by radiant heat and their temperatures were recorded periodically to ensure that they were within a reasonable range (35–38° C).

Myography. The contraction of the muscle studied was recorded with an isometric myograph by means of a mechano-electric transducer valve (RCA 5734), the output of which was displayed on a cathode ray oscilloscope. The linearity of this recording system was confirmed in the first place by calibration with weights, but in addition in most experiments it was checked by using the myograph to record the tension in a spring which was being extended linearly with time (Matthews, 1959). The compliance of the myograph and its supports was less than 0·05 mm/kg. The leg of the cat was held horizontal by steel pins in each end of the tibia, and the compliance of these with their supports was also less than 0·05 mm/kg. The tendon of the muscle was impaled by a steel hook, which was tied in if necessary, and this was connected to the myograph by a rod of thick steel wire. The joint between the hook and the rod, and the rod and the myograph was merely a 'hook-in' chain linkage. The initial tension applied to the muscle was usually about 50 g, which was enough to pull the connecting links taut. Measurement showed that the muscles were then within

their physiological range of length. The arrangement was thus 'rigidly isometric', but it did not prevent small movements of the muscle occurring when it contracted. These consisted in a small change in the direction of pull, arising because the resultant of the forces acting when the muscle was at rest (passive tension in muscle, attached fascia and skin overlying muscle, and gravity acting on connecting wire and on muscle) acted in a slightly different direction from the resultant of the forces acting when the muscle was active. This was not entirely eliminated even when the angle of pull of the muscle on the myograph was altered, but the end of the tendon never moved more than about 0·5 mm laterally. Such movements might well interfere with the strict addition of twitch tensions in the search for polyneuronal innervation, but they would not vitiate some of the other observations which have been made in the present experiments.

Electrical stimulation and recording. Conventional methods were used. The stimulator provided square waves of 0·1 msec duration and these were delivered through a 1:1 isolating transformer and bipolar silver wire electrodes (separation about 4 mm, cathode nearer to muscle than anode). Two stimuli of variable time interval were available and could be delivered either down two separate channels or down the same channel. When they were delivered through separate stimulating electrodes it was found by recording that an inappreciable fraction (less than 0·1 %) of one stimulus appeared at the stimulating electrodes of the other channel. In the initial experiments the stimulus interval was determined by reading a calibrated dial, but in most experiments the interval was determined more directly by displaying the stimuli, together with time marks, upon one beam of one of the two cathode ray tubes used.

Nerve action potentials were recorded through bipolar silver wires; the muscle action potentials were recorded with belly–tendon leads. Amplification was by a push-pull capacity-coupled pre-amplifier, followed by a main amplifier. The over-all frequency response of the system was flat from 10 c/s to 2 kc/s, but in a few experiments the upper limit was increased to 5 kc/s. The linear range of the recording system was determined by applying calibrating pulses of variable size, and the amplification of the action potentials was varied in different experiments so that they were recorded within the linear range of the system.

Two cathode ray oscilloscopes were available for display. They could be photographed simultaneously and allowed both high and low speed events to be recorded together.

Choice of filaments. In each cat the interactions of several different subdivisions of ventral roots were investigated, and altogether 68 such pairs were studied. In seven cats the fibres to the muscle studied were distributed in two different ventral roots and these were then studied entire before being subdivided into filaments for further testing. In the other preparations most of the fibres to the muscle studied lay in a single root and subdivision of the root was the initial procedure. The filaments studied were chosen to give contractions of the same order of size, and no pairs of filaments were used which gave contractions differing by more than a factor of three. Subdivisions producing twitch contractions of less than about 30 g tension were rejected. In order to avoid reduplicating results, the study of interaction between filaments was confined to subdivisions of the same larger filament. Fine filaments derived from separate larger filaments were not compared because any overlap of innervation between them should already have been tested when the interaction between the larger parent filaments was examined.

Sources of error

Spread of current. Spread of stimulating current from one ventral root to the other occasionally occurred and then inevitably mimicked polyneuronal innervation. Current spread could be recognized, however, by determining the relation between the strength of the stimulus and the size of the resulting contraction. Those fibres stimulated directly had thresholds of 50–150 mV (range usually less in any single experiment), and when the stimulus was increased above this the contraction remained constant until with strong shocks

the current spread and the contraction became larger again. In testing for polyneuronal innervation stimuli of about twice maximal strength were used. In a few cases 'current spread' was found for such stimuli, and these preparations were then rejected, as also were those in which the threshold of the directly stimulated fibres was unduly high. In these cases the filaments studied were usually rather small.

Variability of response. The twitch contractions of the muscles did not always remain completely constant. Frequently after starting stimulation the twitch became progressively larger (staircase effect, cf. Eccles & O'Connor, 1939, p. 86). To minimize such effects both stimuli were applied together at a rate of 1/sec until a contraction of constant size was obtained. The experimental observations on any two roots (A and B) were then taken in the order $A+B$, A, B, B, A, $A+B$, and the six oscilloscope traces superimposed on a single photographic record. During recording the rate of stimulation was 1/sec and this was continued (for $A+B$) while photographic paper was moved on between frames. The records on successive frames did not always agree precisely, and in this case the mean result of 2 or 3 frames was taken, but this biological variability remained a significant factor in limiting the accuracy of the experiments. It should be noted that such small absolute errors tended to be magnified by the method of expressing the results as a percentage of the smaller contraction. For example, a variability of 0·5 mm (records measured to 0·1 mm) in a double contraction of height 40 mm composed of two independent twitches of height 30 and 10 mm, becomes a variable tension deficit of 5%. In the first six experiments these errors were slightly larger than in the later experiments, for the different contractions were recorded independently, with short intervals between the observations, instead of being superimposed upon a single record.

The tetanic contractions were always recorded separately on slowly moving paper. Each reading was repeated two or three times and an interval of about 5 sec left between successive observations. The duration of the stimulation was about 1 sec.

RESULTS

Tension overlap

Twitch contractions. On stimulating a pair of ventral root filaments with single shocks, a tension deficit was frequently found on comparing the twitch produced by simultaneous stimulation of the filaments with the sum of the twitch tensions developed by stimulating each filament alone. Typical examples of contractions with tension deficits are shown in Fig. 1 for each of the three muscles studied. The top trace consists of the double response, and the lower two traces show the responses to stimulation of each filament alone. In accord with Hunt & Kuffler (1954), the tension deficit measured at the peak of the twitch has been expressed as a percentage of the tension developed by the smaller contraction. In the three examples shown the deficits, expressed in this manner, were 25, 20 and 14%, and if there were no other factors to consider this could be taken as a measure of the overlap of innervation in these particular cases.

Figure 2 shows the sixty-eight results obtained in the course of all the experiments on the three muscles studied. The mean tension deficit was 11·0%, with a standard deviation of 10·2%. This differs significantly from zero (standard error of mean = 1·2, $t = 9{\cdot}0$, $P < 0{\cdot}001$). In the

absence of any other explanation, therefore, the figure of 11% could be taken to show that polyneuronal innervation was common. Twitch contractions, however, cannot be reliably used in testing for polyneuronal innervation, as even when a rigidly isometric myograph is used appreciable internal shortening of the muscle still occurs, because the contractile part

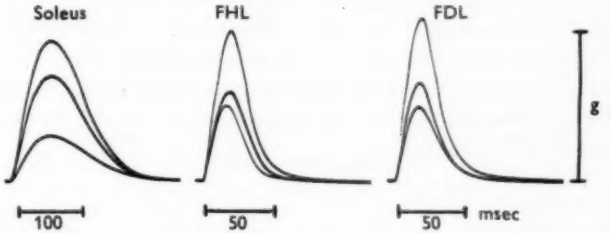

Fig. 1. Myographic records showing typical small 'tension deficits' of the twitch contractions due to simultaneous stimulation of a pair of ventral root filaments. Results from each of the three muscles studied. In each case the top trace is due to the simultaneous stimulation of the two filaments, and the two lower traces are due to their individual stimulation. (The tension scales are different for each muscle. The calibration bar is equivalent to 400 g for soleus, 1120 g for FHL and 110 g for FDL.)

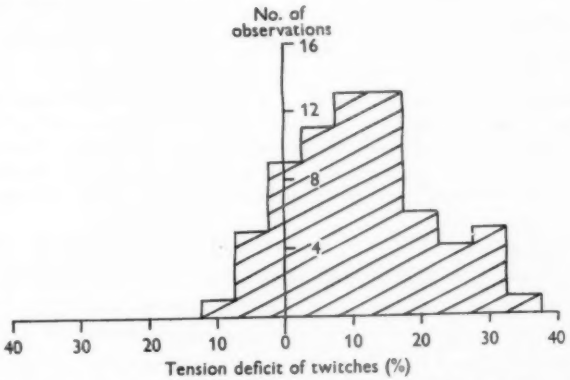

Fig. 2. Histogram of the values of the tension deficits of the twitch contractions found in all the experiments. The observations were made with 68 pairs of ventral root filaments, and include those obtained on each of the three muscles studied. (Any tension *excess* is plotted to the left of zero.)

of the muscle extends the non-contractile 'series elastic component' of the muscle (Hill, 1949, 1951, 1953). If part of the series elastic component were common to the muscle fibres excited by stimulating different ventral root filaments, then the extra shortening occurring with the double response might by itself cause a tension deficit. This may be simulated experimentally by interposing a spring between the muscle and the

myograph, and a typical effect is shown in Fig. 3. With 'isometric' recording the tension deficit was 23 % but on inserting a spring sufficient to reduce the contraction to one quarter of its previous value the tension deficit rose to 65 %. (The isometric twitch tension was about a quarter of the tetanic tension.) Such results were obtained with all three muscles with springs of various strengths (170, 90, 48, 30 and 12 g/mm). The precise value of the tension deficit, however, depended upon both the strength of the spring and the initial tension. Thus it is possible that part if not all of the tension deficits found on recording twitches under isometric conditions depended upon the mechanical properties of muscle rather than upon polyneuronal innervation.

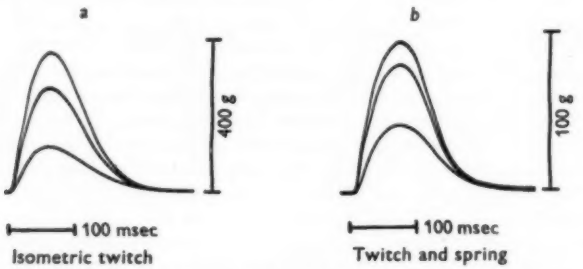

Fig. 3. The effect on the addition of the twitch contractions of using a compliant recording system; superimposed responses of soleus to stimulation of two ventral root filaments either alone or together. *a* was recorded directly with an isometric myograph. *b* was recorded with the same initial tension when a spring of stiffness 48 g/mm was inserted between the tendon and the myograph. The compliant recording increased the tension deficit, and it is therefore possible that the initial tension deficit may have been due to compliance existing within the muscle.

Tetanic contractions. The series elastic component should have a much smaller effect on the interaction of tetanic contractions than upon the interaction of twitch contractions. For a tetanic contraction continues long enough for shortening of the contractile elements to cease, so that each individual muscle fibre can develop the full tension of its active state, and this is unlikely to alter greatly with small changes in the length of the muscle. Any tension deficit due to overlap of innervation between a pair of root filaments should, however, be displayed in either mode of contraction. The tetanic contractions elicited by repetitive stimulation of the pairs of root filaments were, therefore, also recorded in every case. The frequency of stimulation was sufficient to produce a fused tetanic contraction, and was about 30/sec for soleus and about 70/sec for the two other muscles. Figure 4 shows superimposed tracings of tetanic contraction obtained by using the same combinations of ventral roots as those used to obtain the twitch contractions illustrated in Fig. 1. In contrast to the

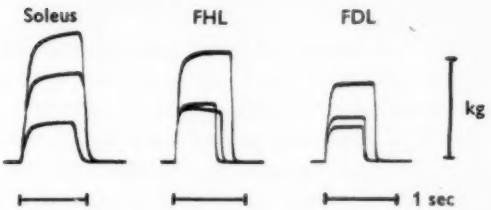

Fig. 4. Tetanic contractions showing no tension deficit, even though the twitch contractions elicited by stimulation of the same combinations of filaments did so (Fig. 1). The responses to stimulation of the filaments alone (lower two traces) or together (upper trace) were recorded individually, and have been superimposed by tracing the original records. (The tension scales are different for each muscle; the calibration bar is equivalent to 1·3 kg for soleus, 4·0 kg for FHL and 0·70 kg for FDL.)

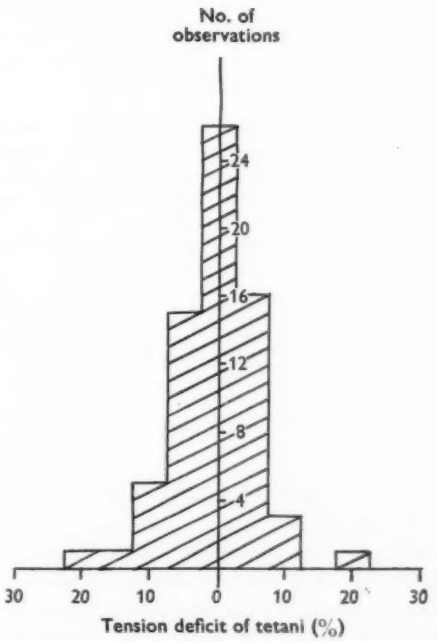

Fig. 5. Histogram of the values of the tension deficits of the tetanic contractions. The observations were made using the same 68 pairs of ventral root filaments as were used to obtain the results shown in Fig. 2. (The tension deficits are plotted to the right of zero. The tension was measured 0·5 sec after the beginning of the contraction.)

results obtained with the twitch contractions the tetanic contractions show no tension deficit at all.

The results of the interaction of the tetanic contractions elicited by stimulation of all the sixty-eight root pairs studied are shown in Fig. 5. The mean value of the tension deficit was $-0{\cdot}6\,\%$ (i.e. a 'tension excess'), and this does not differ significantly from zero (S.D. $6{\cdot}0\,\%$). Thus, this mode of testing for overlap of innervation between the roots provides no evidence at all for the existence of polyneuronal innervation. Nor was there any significant correlation between the tension deficits of the twitch and tetanic contractions elicited by stimulation of each pair of filaments (correlation coefficient 0·18, which does not differ significantly from zero), which suggests that the values found in the two cases depended upon different factors.

Comparison of the results obtained on the three muscles. Pooling of the results obtained from three different muscles might have obscured a significant effect which was peculiar to one muscle. This does not seem to have happened, as may be seen by comparing the various statistics

TABLE 1. Various statistics possibly indicative of polyneuronal innervation calculated for each muscle alone. In each case the value of the mean is given, followed by the standard deviation of the sample. Each value is expressed as a percentage of the smaller of the tensions produced by stimulation of the ventral root filaments independently. The two negative values of the tension deficit represent 'tension excesses'. The contraction rise was the maximum increase in the contraction found on increasing the interval between the stimuli delivered to the two filaments from zero to over 15 msec

Muscle	No. of observations	Twitch deficit	Tetanus deficit	Contraction rise
Soleus	38	$7{\cdot}4 \pm 8{\cdot}9$	$0{\cdot}7 \pm 4{\cdot}7$	$0{\cdot}4 \pm 1{\cdot}2$
Flexor hallucis	18	$17{\cdot}5 \pm 10{\cdot}6$	$0{\cdot}2 \pm 6{\cdot}5$	$3{\cdot}0 \pm 1{\cdot}3$
Flexor digitorum	12	$12{\cdot}8 \pm 9{\cdot}4$	$-5{\cdot}8 \pm 6{\cdot}9$	$1{\cdot}3 \pm 2{\cdot}5$
Total	68	$11{\cdot}0 \pm 10{\cdot}2$	$-0{\cdot}6 \pm 6{\cdot}0$	$1{\cdot}2 \pm 2{\cdot}7$

calculated for each muscle separately and shown in Table 1. In each case there was a tension deficit for the twitch contractions which differed significantly from zero (t test, $P < 0{\cdot}001\,\%$), but in no case was there a significant tension deficit for the tetanic contractions. (The mean 'tension excess' for the tetanic contractions of flexor digitorum longus is significant at the $5\,\%$ level but not at the $1\,\%$ level.) For flexors hallucis and digitorum there was no correlation between the values of the tension deficits found by using twitch and tetanic contractions for each combination of filaments. For soleus there was such a correlation ($n = 0{\cdot}56$, $P < 0{\cdot}001\,\%$), but this might have depended upon mechanical factors.

Summation of the electrical activity of muscle

In most of the experiments upon soleus evidence for overlap of innervation between the pairs of rootlets was also sought by recording the electromyogram with belly–tendon leads. The potential recorded was polyphasic and lasted about 10 msec (cf. Eccles & O'Connor, 1939). The electrical responses elicited by independent and simultaneous stimulation of a pair of filaments are shown in Fig. 6, where it can be seen by naked-eye inspection that no gross occlusion occurred. This has been the invariable result in studying the electromyogram upon stimulation of 26 pairs of filaments in nine cats. Attempts to detect small degrees of overlap by measurement of the records have been somewhat unsatisfactory, but again they have failed to provide any evidence in favour of the existence

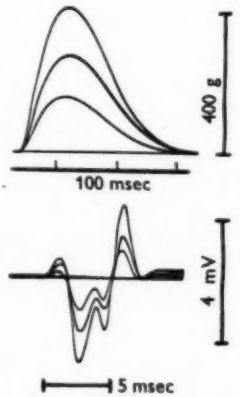

Fig. 6

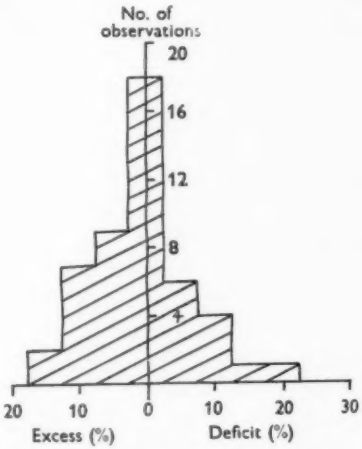

Fig. 7

Fig. 6. Comparison of the electromyograms elicited by separate and by combined stimulation of two ventral root filaments. Above, twitch contraction of soleus; below, the electrical activity of soleus recorded with belly–tendon leads (note base line in both cases). The twitch contractions show a tension deficit of 12% on combined stimulation, but this is not found in the electromyogram.

Fig. 7. Histogram of the values of the 'potential deficits' found in studying the electromyogram of soleus. Forty-eight observations derived from stimulation of 26 pairs of filaments. The values plotted are those obtained by measuring each major peak of each electromyogram. (The electromyograms elicited by stimulation of either root alone were usually similar, and the measurements were made at peaks in order to reduce the errors of measurement. Peaks have been excluded in which the smallest potential gave a deflexion of less than 1 mm on the recording paper, as these could not be measured with sufficient accuracy. This arbitrary selection was made because the value of the 'potential deficit' was not always completely constant for the whole duration of the electromyogram.)

of polyneuronal innervation. The amplitude of each of the potentials was measured at various times after the stimuli, and for each time any difference between the sum of the individual potentials and the combined potential was expressed as a percentage of the smaller of the individual potentials to give the 'potential deficit'. It was found, however, that even in a single record the value of the 'potential deficit' was not constant at different times after the stimulus. For example, in Fig. 6 there is a 'potential deficit' of 4% for the second peak, and 'potential excesses' of 3 and 11% for the third and fourth peaks, respectively. It is possible that this variability depended upon interaction between the action potentials of different muscle fibres, for then the propagation of the action potential along some fibres might be slowed, while along others it might be accelerated (Katz & Schmitt, 1940; Merton, 1954). The degree of synchronization of the action potentials of different muscle fibres would then be slightly altered, so that simple addition of the electromyogram could not be expected. (This would probably cause a 'potential excess', but reasons for expecting a 'potential deficit' can also be postulated.)

When results from all the experiments were plotted as a histogram, it was found that 'potential excesses' were slightly commoner than 'potential deficits' (Fig. 7; note arbitrary selection of points as described in legend). The mean value of these observations was $\pm 1{\cdot}0\%$ and this does not differ significantly from zero (S.D. $\pm 7{\cdot}2\%$, 48 observations). The study of the extent of summation of the electromyogram has, therefore, produced no evidence in favour of the existence of polyneuronal innervation. But we do not feel confident that a small degree of overlap would have been detected by these means. Nor were these experiments performed upon flexors digitorum and hallucis, because when one was studied the other remained innervated, so that any potential recorded with belly–tendon leads would have been derived from both muscles.

Testing for polyneuronal innervation by altering the stimulus interval

Any tension deficit due to polyneuronal innervation depends ultimately upon the 'all-or-nothing' behaviour of individual muscle fibres. For if any particular muscle fibre is supplied by two different nerve fibres and these are activated simultaneously, then the muscle fibre should contract no more strongly than when only one of its nerve fibres is activated; it seems reasonable to ignore the possible slight effect on the contraction of activating the two ends of the muscle at the same or at slightly different times (Rushton, 1932; Abbott & Ritchie, 1951; Goffart & Ritchie, 1952). If, however, the two nerve fibres are activated at sufficiently different times the two muscle action potentials which are evoked will each traverse the muscle membrane throughout its length. Such double activation will

cause a 'double twitch' developing appreciably more tension than a single twitch. Cooper & Eccles (1930) found that the ratio of the tensions developed by single and double twitches was 1:1·9 for soleus, and in the present experiments the ratio was usually found to be about 1:2·0 for each of the three muscles, though the precise value found depended upon the particular preparation and the initial tension. Thus, if the interval between the 'simultaneous' stimuli applied to the two rootlets is increased sufficiently to excite any doubly innervated fibres twice, then the contraction should increase in size and this increase should approximately annul any initial tension deficit due to overlap of innervation between the rootlets. This further test for the existence of overlap of innervation was applied to each of the 68 combinations of filaments studied.

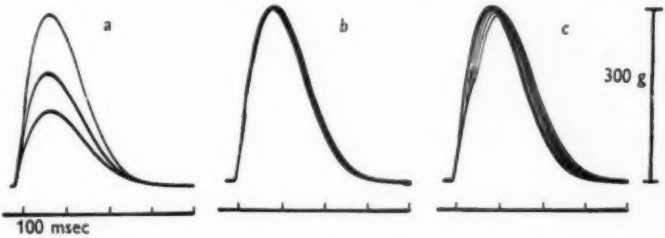

Fig. 8. The effect on the contraction of altering the time interval between the stimuli applied to two ventral root filaments. *a*, twitch contractions elicited by individual and simultaneous stimulation of two ventral root filaments, showing a tension deficit of 22%. *b*, *c*, superimposed traces of the response to stimulation of both roots, with varying time intervals between the two stimuli (*b*, 0, 2, 5, 11, 17 and 22 msec; *c*, 0, 2, 5, 11, 22, 32 and 48 msec). The contraction did not increase in size on increasing the stimulus interval, and it is therefore unlikely that the initial tension deficit can have been due to overlap of innervation between the two filaments.

A typical example of the effect of increasing the stimulus interval is shown in Fig. 8. Figure 8*a* shows the responses to simultaneous stimulation of two rootlets, and to separate stimulation of each rootlet. In this case there was a tension deficit of 22%. Figure 8*b*, *c* shows superimposed responses to stimulation of both roots, but with the interval between the stimuli progressively increased for each trace of each record. In no case was there an increase in the size of the contraction on increasing the stimulus interval, and it therefore appears unlikely that there can have been appreciable overlap of innervation between the two filaments.

Unfortunately the precise stimulus interval required to produce double stimulation of any doubly innervated muscle fibres is not known, though it must be under 20 msec. It will be equal to the muscle refractory period of about 2 msec (Eccles & O'Connor, 1939) plus the unknown conduction

time of the muscle action potential from one end-plate to the other. The maximum conceivable value for this conduction time in soleus would be about 18 msec, corresponding to end-plates lying at each end of the muscle fibre (perhaps 5 cm long) and a conduction velocity of 2·8 m/sec (Eccles & O'Connor, 1939). The true time is likely to be very much shorter, for histological work shows that the end-plates usually lie in the middle of a muscle, and do not occur at the extreme ends of the muscle fibres (Coërs & Woolf, 1959). The time for the two other muscles is likely to be shorter than that for soleus, as it is probable that in these faster muscles the conduction velocity of the action potentials is higher (Eccles & O'Connor, 1939). Too large stimulus intervals must be avoided because the 'twitch' then becomes obviously double and develops less tension, as would be expected (Fig. 8*c*).

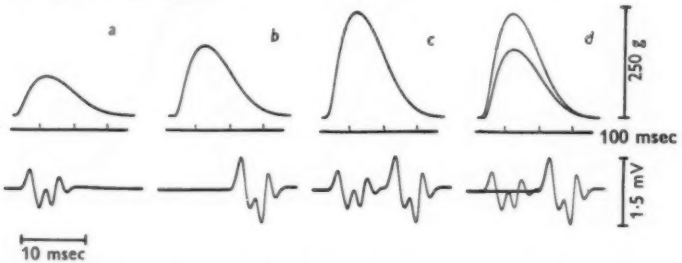

Fig. 9. Testing for the 'effective refractory period' of any doubly innervated muscle fibres. Above, twitch contractions of soleus; below, its electromyogram recorded with belly–tendon leads. *a*, stimulation of one filament alone; *b*, stimulation of other filament alone; *c*, stimulation of both filaments with a stimulus interval of 10 msec; *d*, responses of *b* and *c* superimposed. The electromyogram set up by the second stimulus is the same whether or not it is preceded by the first stimulus, showing that any muscle fibres innervated by both roots are doubly excited. (In this experiment the twitch contraction showed a tension deficit of 10%, and the contraction did not increase when the stimulus interval was increased from 0 to 10 msec.)

In view of this uncertainty it seemed desirable to demonstrate by electromyography that appropriate intervals were being used. When the stimulus interval was increased to 10–15 msec the electrical activity of the two groups of muscle fibres activated by the two stimuli could be completely separated (Fig. 9*a*, *b*, *c*), and it could be seen that the electromyogram elicited by the second stimulus was the same whether or not it was preceded by the first stimulus (Fig. 9*b*, *c*). This could be more conclusively demonstrated by superimposing the two types of response on a single record (Fig. 9*d*; the slight thickening of the trace at the beginning of the second action potential is accounted for by a persistence of the first potential). Thus at this stimulus interval (10 msec) any doubly innervated

muscle fibres must have recovered sufficiently from their activation by the first filament to be normally excited by the second filament. Yet in this experiment there was a contraction deficit of 10% for the twitch contractions, and there was no increase in the size of the combined contraction when the stimulus interval was increased to 10 msec. This deficit therefore cannot have been due to polyneuronal innervation, for it has been demonstrated directly that it does not alter when the stimulus interval

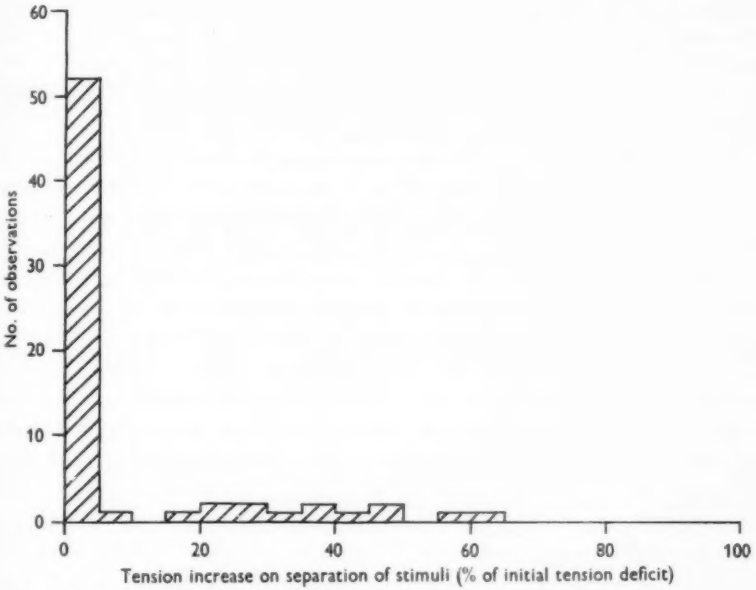

Fig. 10. Histogram of the results obtained on increasing the time interval between the stimuli applied to the 68 pairs of ventral root filaments. Any increase in the twitch contraction on increasing the stimulus interval has been plotted as a percentage of the initial tension deficit. If the initial tension deficit had been due entirely to overlap of innervation it should have been approximately annulled by increasing the stimulus interval, and the mean tension increase should then have been 100% in the above histogram.

is increased sufficiently to produce normal excitation of the second group of muscle fibres. A similar test was performed in slightly over half the experiments on soleus, and in all these it appeared that any second group of end-plates which existed were capable of re-exciting their muscle fibres at intervals less than 10–15 msec after activity of the first group of end-plates.

The increases in the contraction found on increasing the stimulus interval

by up to 15 msec are shown in Fig. 10 for the whole series of experiments. Here any increase in the twitch tension is expressed as a percentage of the initial tension deficit found for the twitch contractions. In 78 % of the cases, as in that illustrated in Fig. 8, there was no significant increase in the contraction. In the remainder there were increases in the contraction but these were small and were never sufficient to annul the initial tension deficit. As already discussed, if the initial tension deficit had been due solely to overlap of innervation it should have been approximately annulled by increasing the stimulus interval, so that these experiments again show that polyneuronal innervation is neither the sole cause nor a common cause of the tension deficits found for the twitches.

If it be supposed that, when it occurred, the increase in the tension produced by increasing the stimulus interval was due to overlap of innervation, then the extent of the rise may be taken as a measure of the degree of overlap by expressing it as a percentage of the smaller of the individual contractions. This gives an approximate value for the percentage of muscle fibres supplied by the smaller root which were also supplied by the larger root, for under the present experimental conditions a 'double twitch' produced about twice the tension of a 'single twitch'. The maximum value found was 10 %. The mean value for the whole 68 observations was only 1·2 %. The mean values for each muscle are given in Table 1. It may be noted that of the 14 occasions in which any rise occurred at all, 8 were for flexor hallucis longus, though only 18 out of 68 pairs of rootlets were from this muscle. For flexor digitorum there were 3 rises out of 12 observations; but for soleus only 3 rises were found in 38 observations, and these rises were not constantly found for these three combinations of filaments (such variability was also found in some of the other observations). The time interval required to produce the maximum contraction varied from 4 to 10 msec in different experiments, but it was not determined at all precisely as the stimulus interval was altered in steps (cf. Fig. 8).

Possible ephaptic stimulation. These occasional small increases in the contraction may, however, have been due to the ephaptic stimulation of the nerve fibres of one filament by the muscle action potential set up by stimulation of the other filament (Lloyd, 1942). For example, in two cases it was found that on increasing the stimulus interval the contraction first decreased below its initial level (interval about 2 msec) before increasing above it (interval over 4 msec). This finding cannot be explained in terms of polyneuronal innervation, but both the initial fall and the subsequent rise can be explained in terms of ephaptic stimulation of motor nerve fibres by the muscle action potential, a phenomenon which is known to occur (Lloyd, 1942; Leksell, 1945; Brown & Matthews, 1960). Falls of tension were not seen otherwise, but ephapses could also cause rises in

tension without initial falls, and such increases cannot, therefore, be attributed unequivocally to polyneuronal innervation.

Owing to the complexity of the situation, increasing the stimulus interval might, depending on the precise circumstances, either increase or decrease the amount of ephaptic excitation taking place. The simplest situation is when either stimulus given alone leads to the ephaptic excitation of some nerve fibres of the other filament. Then if the two stimuli are given together the amount of such ephaptic excitation will decrease, because the nerve fibres will be somewhat refractory to the ephaptic stimulus of the muscle action potential as they will just have been excited by the direct stimulus. In this case, therefore, increasing the stimulus interval will increase the contraction. On the other hand, when the stimuli are given together they will set up a compound muscle action potential which is approximately synchronous in the two groups of muscle fibres, and this large action potential may excite some nerve fibres which would not be ephaptically excited by either of the individual action potentials alone. These marginally excited fibres might either be those which were not stimulated directly, because their axons did not lie in the pair of ventral root filaments being studied, or those which were excited directly and were also being re-excited by the muscle action potential. In both cases increasing the stimulus interval would reduce the ephaptic response and the contraction by 'desynchronizing' the muscle action potential (Fig. 6; Lloyd, 1942; Brown & Matthews, 1960). These two opposite effects on the contraction of increasing the stimulus interval might be expected to operate over different time scales, and if both occurred in the same experiment the two apparently anomalous results already cited would be explained.

In a few experiments direct evidence for the occurrence of ephapses was sought by recording from ventral root filaments. In these, ephaptically generated spikes were sometimes recorded, but in all save one case their latency was too short (< 5 msec) for them to have originated in the muscle studied, and they probably arose either in the hip muscles or at the cut ends of nerves. Even the one spike of longer latency could only have been proved to have originated in the muscle studied by cutting its nerves, and this would have terminated the experiment. In this experiment there was a 'contraction rise', and it is only in such experiments that ephapses would be expected to occur. Unfortunately the small contraction rises occurring in the present experiments could only be recognized retrospectively by inspection of the photographic records, and it did not appear worth while to record from the ventral root in every experiment.

The relation between the nerve action potential and the muscle twitch

A completely different method of testing for the existence of polyneuronal innervation is to make a quantitative study of the response of a muscle to graded stimulation of its nerve. In the absence of polyneuronal innervation the size of the contraction elicited by submaximal stimulation might be expected to be directly proportional to the number of motor fibres excited. But if polyneuronal innervation does exist those motor fibres which are excited last because they are of high threshold will cause relatively little extra tension to be developed, as many of the muscle fibres they supply will already have been excited by other motor fibres. The number of active motor nerve fibres may be estimated to a first approximation by recording their action potential monophasically from the cut end of a ventral root, and measuring its area. (For experimental arrangement and typical records see Brown & Matthews, 1960.) If polyneuronal innervation

were widespread, a graph of twitch tension against action potential area should be a curve flattened at its top, and not a straight line. The construction of such a graph, therefore, provides another possible test for the existence of polyneuronal innervation.

The assumptions involved in expecting a linear relation to be found in the absence of polyneuronal innervation are as follows.

1. No ephaptic excitation of nerve fibres by the muscle action potential should be taking place. This is particularly liable to occur when a large part of the muscle is activated synchronously (Lloyd, 1942; Brown & Matthews, 1960), and could have the effect of increasing the slope of the top part of the relation because some muscle fibres would then be doubly excited (i.e. unduly great tension would be produced by each motor fibre, because in addition to activating its own muscle fibre it would also be assisting in stimulating other fibres). In four experiments no significant 'back-response' of ephaptic impulses was found in the ventral root, and it is probable that in these preparations little or no ephaptic excitation was occurring in soleus. But in two other experiments there was a 'back-response', and the graph departed from a linear relation in the manner expected.

2. The ventral root fibres in which the action potential was recorded should be a random sample of the motor fibres supplying the muscle. If they were not, the linear relation might be distorted in any manner, depending upon the selection of the fibres. A random selection seems likely since the linear relation was found in more than one experiment. In addition, in each case over half the motor fibres to soleus were recorded from, as judged by the tension produced by their stimulation.

3. The twitch tensions generated by the different muscle fibres should add arithmetically. All the previous evidence of the present paper suggests that this is not strictly true, but the effect is probably not large enough to be displayed in the present experiment.

4. There is a random relation between the size of a nerve fibre (and hence the size of its action potential), and the number of muscle fibres it innervates (hence the twitch tension of the motor unit). Alternatively it may be assumed that the twitch tension of a motor unit is directly proportional to the diameter of its nerve fibre, for the area of the monophasically recorded action potential would appear to be proportional to the fibre diameter (height proportional to diameter, duration independent of diameter, Gasser & Grundfest, 1939).

5. There must be no group of nerve fibres which contributes to the action potential but which does not contribute to the twitch tension. The action potential of the γ motor fibres could usually be recognized in the present experiments and was not included in the estimate of action potential area. (It was anyhow too small to be significant.) Also care was taken to dissect the branches of the nerve to gastrocnemius well clear of the nerve to soleus, and tripolar stimulating electrodes, with central cathode, were used with the object of further avoiding current spread to the gastrocnemius fibres. It may here be noted that where the stimulating electrodes were applied to the nerve, some branching of nerve fibres may already have occurred (Eccles & Sherrington, 1930). This should not, however, have affected the experiment, for if one branch of an axon were excited, the impulse would invade the other branch also, after being conducted antidromically in the first branch.

Figure 11 shows the result of an experiment upon the soleus muscle. It will be seen that the points do indeed fall upon a straight line. The grouping of the points is probably a result of the coarseness of the potentiometer controlling the intensity of the stimulus. A linear relation was found also in the three other experiments in which there was no 'back-response'. These results suggest both that polyneuronal innervation cannot be wide-

spread and that the various assumptions involved in expecting a linear relation are approximately correct. Of course if any overlap of innervation were not random, but occurred only between nerve fibres of similar threshold, then its existence could not be detected in this experiment. In addition it is conceivable that any effects produced by polyneuronal innervation were obscured by some other factor. The experiment, therefore, certainly does not completely exclude the existence of polyneuronal innervation, but it has failed to provide the evidence in its favour which might have been expected if polyneuronal innervation were common.

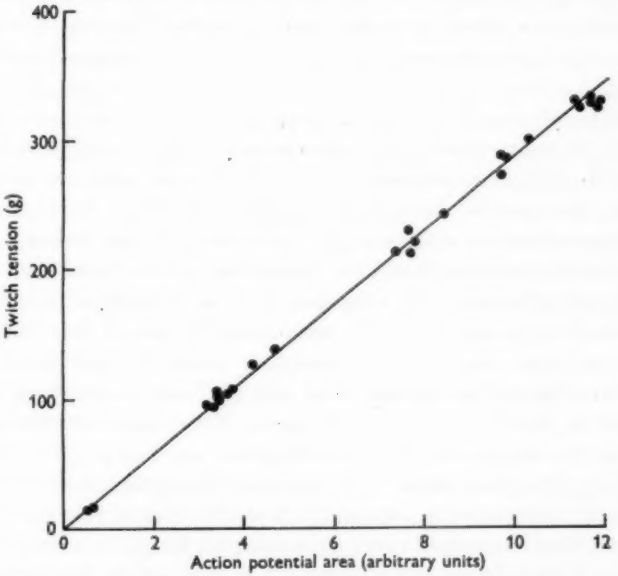

Fig. 11. The relation between the size of the twitch contraction of soleus and the size of the compound action potential of its motor fibres, on altering the strength of a stimulus applied to its nerve. Ordinate, peak tension of twitch in grams; abscissa, area of the action potential recorded monophasically from an appropriate ventral root (arbitrary units; a 1 mV square wave of 1 msec duration would have an area of 34·2 of these units). Dots, experimental points, with a straight line drawn through them by eye.

DISCUSSION

In the present study several relatively independent methods have failed to produce any consistent evidence for the existence of polyneuronal innervation in the three muscles studied. The tension overlap which was found for the twitch contractions was probably largely due to mechanical factors, for it was not found in studying tetanic contractions elicited by stimulation of the same pairs of root filaments. The occasional effect on

the twitch contraction of increasing the interval between the stimuli seems as likely to have been due to ephaptic stimulation of nerve fibres as to an overlap of innervation. Even if it be supposed that this was entirely due to overlap of innervation the average overlap was only 1·2 %, and this cannot mean that many muscle fibres are doubly innervated. It should be noted, however, that the determination of the degree of overlap of innervation between pairs of ventral root filaments is not a particularly sensitive method for the detection of polyneuronal innervation, because any doubly innervated muscle fibres must be supplied in some cases by axons running in the same ventral root filament. The relation between the size of the nerve action potential and the size of the twitch contraction again provided no evidence in favour of polyneuronal innervation. If some end-plates had been spontaneously in a state of neuromuscular block then their existence would not have been detected by any of the present experiments; but as the muscles were *in situ* with a normal circulation it is improbable that neuromuscular block could have been common enough to distort the results. It appears unlikely, therefore, that polyneuronal innervation exists on a large scale, but owing to the limitations of the methods used its existence on a small scale has certainly not been excluded.

This conclusion contrasts with that of Hunt & Kuffler (1954), who, on the basis of experiments on the same muscles, stated that polyneuronal innervation was common. We consider, however, that their evidence cannot be accepted as indicative of polyneuronal innervation until it is supported by further control experiments. They used only the method of measuring the tension deficit on simultaneous and independent stimulation of two pairs of roots or subdivision of a root. With this method they studied only twitch contractions, and did not study tetanic contractions. They considered that mechanical factors could play no part in their results, on the basis of a single control experiment in which they found 'practically complete summation' of the twitch contractions of the two heads of gastrocnemius on stimulating the nerves to the two heads of gastrocnemius. In this case there might have been relatively little sharing of the 'series elastic component' between the two groups of fibres, so that this is not a complete control for the absence of mechanical factors when the two groups of muscle fibres are intermingled in a single muscle. It would be surprising, however, if mechanical factors were the sole cause of the large tension deficits ('often' 60–90 %) which they reported. It is possible, however, that Hunt & Kuffler used experimental conditions which encouraged the ephaptic stimulation of nerve fibres by the muscle action potential, for this could cause substantial tension deficits. Ephapses are influenced by both the type and the depth of anaesthesia (Granit, Leksell & Skoglund, 1944; Brown & Matthews, 1960) but Hunt & Kuppler did not

mention the anaesthetic used. Nor did they say what precautions were taken to recognize any tension overlaps occurring as a result of accidental current spread. If we had found the large tension deficits which they described (up to 90%) it should have been possible to decide whether or not they were indeed due to polyneuronal innervation; since twitch tension deficits due to the mechanical effects of the series elastic component should be absent from the corresponding tetanic contractions, and should persist on increasing the stimulus interval. Tension deficits due to massive ephaptic excitation should be recognizable by recording from the ventral roots, and could probably be largely abolished by deep anaesthesia. At any rate it is clear that there are other possible causes for the tension deficits which Hunt & Kuffler observed, and until these are excluded their conclusion cannot be accepted.

Finally, we hope that the present study may help to emphasize the existence of various artifacts which can hinder the detection of small amounts of polyneuronal innervation. Whether or not polyneuronal innervation normally exists at all in limb muscles therefore remains to be decided, and for this some more crucial type of experiment appears to be necessary. Micro-electrode studies probably offer the best hope of establishing the existence of polyneuronal innervation, but their use to disprove its occurrence might be laborious and possibly inconclusive. Meanwhile it seems reasonable to suppose that in mammalian limb muscles polyneuronal innervation does not exist on a large enough scale to be physiologically important.

SUMMARY

1. Experiments were performed on the soleus, flexor digitorum and flexor hallucis muscles of anaesthetized cats in order to search for evidence indicating the existence of polyneuronal innervation of individual muscle fibres. Appropriate pairs of ventral roots or ventral root filaments were stimulated and the muscle contraction recorded.

2. When twitch contractions were studied a small 'tension deficit' was found on average on comparing the tension developed by simultaneous stimulation of the two filaments with the sum of the tensions developed by their individual stimulation.

3. When in the same experiments tetanic contractions were studied there was on average no tension deficit at all. The difference between the two modes of stimulation may have arisen because the strict addition of the twitch contractions was prevented by yield of the 'series elastic component' of muscle, for much larger twitch deficits were found when a spring was inserted between the muscle and the myograph.

4. There was on average no 'potential deficit' in the electromyogram of soleus elicited by simultaneous stimulation of two filaments, when it was

compared with the sum of the electromyograms produced by individual stimulation of the two filaments.

5. When the time interval between the stimuli applied to the two filaments was increased from 0 to 20 msec there was usually no increase in the size of the contraction. Such an increase would have been expected if there had been any muscle fibres innervated by both roots. In a few cases small tension increases were found, which might have been attributable to the existence of polyneuronal innervation, but these appear equally likely to have been due to ephaptic stimulation of a few fibres of one filament by the muscle action potential set up by stimulation of the other filament.

6. In some experiments the action potential of the nerve fibres of soleus was recorded monophasically from a ventral root on stimulating the muscle nerve. When the strength of the stimulus was varied it was found that a graph of the area of the action potential against the size of the twitch was approximately linear. Reasons are given for supposing that such a result would not have been obtained if many muscle fibres had been innervated by more than one nerve fibre.

7. It is concluded that no consistent evidence has been obtained for the existence of polyneuronal innervation, but the possibility of its occurrence on a small scale has not been excluded.

We wish to thank Mr E. T. Giles for technical assistance.

REFERENCES

ABBOTT, B. C. & RITCHIE, J. M. (1951). The onset of shortening in striated muscle. *J. Physiol.* **113**, 336–345.

AGDUHR, E. (1916). Morphologischer Beweis der doppelten (plurisegmentalen) motorischen Innervation der einzelnen quergestreiften Muskelfasern bei den Säugetieren. *Anat. Anz.* **49**, 1–13.

BROWN, M. C. & MATTHEWS, P. B. C. (1959). Further evidence relating to the possible existence of polyneuronal innervation of muscles of the hind-limb of the cat. *J. Physiol.* **149**, 46–47*P*.

BROWN, M. C. & MATTHEWS, P. B. C. (1960). The effect on a muscle twitch of the back-response of its motor nerve fibres. *J. Physiol.* **150**, 332–346.

CATTELL, M. (1928). Plurisegmental innervation in the frog. *J. Physiol.* **66**, 431–442.

COËRS, C. & WOOLF, A. L. (1959). *The Innervation of Muscle.* Oxford: Blackwell.

COOPER, S. & ECCLES, J. C. (1930). Isometric recordings from mammalian muscles. *J. Physiol.* **69**, 377–385.

DENNY-BROWN, D. & PENNYBACKER, J. B. (1938). Fibrillation and fasciculation in voluntary muscle. *Brain*, **61**, 311–334.

ECCLES, J. C. & SHERRINGTON, C. S. (1930). Numbers and contraction-values of individual motor-units examined in some muscles of the limb. *Proc. Roy. Soc.* B, **106**, 326–357.

ECCLES, J. C. & O'CONNOR, W. J. (1939). Responses which nerve impulses evoke in mammalian striated muscles. *J. Physiol.* **97**, 44–102.

FEINDEL, W., HINSHAW, J. R. & WEDDELL, G. (1952). The pattern of motor innervation in mammalian striated muscle. *J. Anat.* **86**, 35–48.

FULTON, J. F. (1926). *Muscular Contraction and the Reflex Control of Movement.* London: Baillière, Tindall and Cox.

GASSER, H. S. & GRUNDFEST, H. (1939). Axon diameters in relation to the spike dimensions and the conduction velocity in mammalian A fibres. *Amer. J. Physiol.* **127**, 393–414.

GOFFART, M. & RITCHIE, J. M. (1952). The effect of adrenaline on the contraction of mammalian skeletal muscle. *J. Physiol.* **116**, 357–371.

GRANIT, R., LEKSELL, L. & SKOGLUND, C. R. (1944). Fibre interaction in injured or compressed region of nerve. *Brain*, **67**, 125–140.

HILL, A. V. (1949). The abrupt transition from rest to activity in muscle. *Proc. Roy. Soc.* B, **136**, 399–420.

HILL, A. V. (1951). The effect of series compliance on the tension developed in a muscle twitch. *Proc. Roy. Soc.* B, **138**, 325–329.

HILL, A. V. (1953). The mechanics of active muscle. *Proc. Roy. Soc.* B, **141**, 104–117.

HINSEY, J. C. (1934). The innervation of skeletal muscle. *Physiol. Rev.* **14**, 514–585.

HOFFMAN, H. (1951*a*). Fate of interrupted nerve-fibres regenerating into partially denervated muscles. *Aust. J. exp. Biol. med. Sci.* **29**, 211–219.

HOFFMAN, H. (1951*b*). A study of the factors influencing innervation of muscles by implanted nerves. *Aust. J. exp. Biol. med. Sci.* **29**, 289–307.

HUNT, C. C. & KUFFLER, S. W. (1954). Motor innervation of skeletal muscle: Multiple innervation of individual muscle fibres and motor unit function. *J. Physiol.* **126**, 293–303.

IWASAKI, S. (1957). Mononeural double innervation of an amphibian striated muscle fibres. *Jap. J. Physiol.* **7**, 267–275.

JARCHO, L. W., EYZAGUIRRE, C., BERMAN, B. & LILIENTHAL, J. L. (1952). Spread of excitation in skeletal muscle: some factors contributing to the form of the electromyogram. *Amer. J. Physiol.* **168**, 446–457.

JEFFERSON, A. (1954). Aspects of the segmental innervation of the cat's hind limb. *J. comp. Neurol.* **100**, 569–596.

KATZ, B. & KUFFLER, S. W. (1941). Multiple motor innervation of the frog's sartorius muscle. *J. Neurophysiol.* **4**, 209–223.

KATZ, B. & SCHMITT, O. H. (1940). Electric interaction between two adjacent nerve fibres. *J. Physiol.* **97**, 471–488.

KUFFLER, S. W. & VAUGHAN WILLIAMS, E. M. (1953). Small-nerve junctional potentials. The distribution of small motor nerves to frog skeletal muscle, and the membrane characteristics of the fibres they innervate. *J. Physiol.* **121**, 289–317.

LEDERER, R. & LEMBERGER, F. (1907). Zur Frage der doppelten Innervation von Muskeln des Warmblüters. *Pflüg. Arch. ges. Physiol.* **119**, 95–109.

LEKSELL, L. (1945). The action potential and excitatory effect of the small ventral root fibres to skeletal muscle. *Acta physiol. scand.* **10**, Suppl. 31.

LLOYD, D. P. C. (1942). Stimulation of peripheral nerve terminations by active muscle. *J. Neurophysiol.* **5**, 153–165.

MATTHEWS, P. B. C. (1959). The dependence of tension upon extension in the stretch reflex of the soleus muscle of the decerebrate cat. *J. Physiol.* **147**, 521–546.

MERTON, P. A. (1954). Interaction between muscle fibres in a twitch. *J. Physiol.* **124**, 311–324.

QUEDNAU, W. (1926). Plurisegmentelle Innervation. *Pflüg. Arch. ges. Physiol.* **212**, 541–546.

RUSHTON, W. A. H. (1932). Nerve supply to Lucas's alpha substance. *J. Physiol.* **74**, 231–261.

SHERRINGTON, C. (1930). Some functional problems attaching to convergence. *Proc. Roy. Soc.* B, **105**, 332–362.

STEVENS, H. C. & KARRER, E. (1929). The measurement of muscular tension and its bearing on pluri-axonal innervation. *Amer. J. Physiol.* **88**, 686–697.

TIEGS, O. W. (1953). Innervation of voluntary muscle. *Physiol Rev.* **33**, 90–144.

J. Physiol. (1960), **151**, *pp.* 458–471
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SODIUM TRANSPORT BY THE CHORIOALLANTOIC MEMBRANE OF THE PIG

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It has been known for many years that the foetal urine and allantoic fluid may closely resemble each other in composition, and that both may be hypotonic to the plasma, primarily as a result of their lower concentrations of sodium and chloride ions (Jacqué, 1903, 1906; Alexander, Nixon, Widdas & Wohlzogen, 1958). Owing to this and to the anatomical connexion between the bladder and the allantoic sac via the urachus, it has frequently been suggested that the fluid in the sac consists largely of the urine secreted by the foetus. This view has been criticized, particularly as it applies to the earlier stages of gestation (Wislocki, 1935; McCance & Dickerson, 1957), and it has recently been shown (Stanier, 1960) that the urine found in the bladder of the foetal pig at the 46th day of gestation is isotonic with the plasma, whereas the allantoic fluid is very hypotonic and has become so since the 20th day. It has furthermore been demonstrated that the fluid at this age has a pH of about 6, due to the presence in it of much carbonic acid, that it contains ammonia and that its membranes display carbonic anhydrase activity (Widdowson & McCance, 1958; McCance & Widdowson, 1960) and also glutaminase activity (Radde & McCance, 1959). These observations, taken together, suggested that the membrane might be endowed with unusual permeability characteristics and might be capable of active ion transport. This report is concerned with a preliminary investigation of these properties.

METHODS

The pigs were obtained from the A.R.C. Unit of Reproductive Physiology and Biochemistry of the University of Cambridge, or were reared at the laboratory farm. The majority were killed between 40 and 50 days of gestation, though the range was from 18 to 67 days. The membranes were obtained by incising the uterus directly opposite the broad ligament in the area overlying the head of one foetus. Here the chorioallantoic membrane is almost free from visible blood vessels. A piece was excised approximately 3×3 cm^2 consisting of all the uterine layers and the opposed chorioallantoic membrane, taking care to avoid the area of fusion with the amnion. After rinsing in warm bathing medium, the tissue was pinned with the foetal side uppermost to a block of high-melting-point paraffin wax,

submersed in bathing medium contained in a shallow glass vessel. Ordinarily, the next manœuvre was to seize a corner of the chorioallantoic membrane with smooth forceps and to reflect the surface of the membrane gently on itself. As this was done, loose adhesions between the chorioallantoic membrane and the endometrium tended to lift the epithelium from the muscle layer. With a second pair of fine-toothed forceps, the endometrium was teased away and ultimately completely removed from the chorioallantoic membrane. After 60 days of gestation, when the chorionic epithelium becomes excessively friable, some membranes were obtained with the cleavage plane in the uterine submucosa between endometrium and myometrium.

The chorioallantoic membrane with or without attached endometrium was then transferred to a Petri dish of warm bathing solution. There, in some instances, a second dissection was carried out and a cleavage plane established in the stromal layer separating the chorion from the allantois or in the potential space between the full thickness of the chorioallantoic membrane and the endometrium.

Portions of tissue were next minutely inspected. Those showing any sign of discontinuity in their epithelial surfaces were discarded. The remainder were trimmed to appropriate size and mounted for study by one of two techniques. Some specimens were placed in chambers of the type developed by Ussing (1949) for study of electrical activity. Others were fashioned into bags tied to the ends of 4 cm lengths of thin-walled polythene tubing of 0·15 cm internal diameter and suspended in chambers containing the bathing medium. The surface areas of the almost spherical bags ranged from 2·5 to 3·5 cm^2. This mounting was especially suitable for measuring the passage of water across the membrane in response to osmotic gradients by a gravimetric technique similar to that used by Huf, Parrish & Weatherford (1951) and Bentley (1958).

In experiments with the latter type of mounting, the amount of fluid in the membrane sac was adjusted so that the hydrostatic pressure inside the sac, when immersed, was equal to that outside. The external medium was continuously oxygenated. The internal medium was oxygenated before being placed in the sac but not during the individual periods of observation. Before and at the end of each period, the polythene tubing and the sac with its contents were rapidly weighed in air and the difference between these two weights taken as a measure of the water which had passed across the epithelium. Chemical analyses were performed on the fluid withdrawn from the sac immediately after the weighing which terminated each experimental period. Before the start of the individual periods, the sac was filled and emptied three times with the solution to be studied, and then a pre-determined volume was introduced from a finely calibrated syringe. A number of analyses of the fluid withdrawn from the sac after it had been filled for the third time showed that its sodium and chloride concentrations were identical, within the limits of the errors of the methods, with those of the stock bottles from which it was obtained. The fluid in the large reservoir bathing the external surface of the tissue was completely renewed before each period of observation.

Sodium and potassium estimations were performed on an EEL flame photometer, and chloride was determined by a micro-modification of the method of Shales & Shales (1941). The instrument designed by Hervey (1955) was used for measuring osmolality. Weighings were performed with a torsion balance (capacity 0–1000 mg). A Cambridge Portable pH meter was used for estimation of the potential difference between the two sides of the membranes, after correction for circuit asymmetry. Short-circuit current readings were made with an Acweeco Electronic Testmeter. Dry batteries with a suitable potential divider in series were employed to provide the counter-potential source. The bathing medium used during the dissection, and except where otherwise noted during study of the properties of the tissue, had the following composition (m-equiv/l.): Na, 151; K, 4·7; Ca, 5·0; Mg, 0·21; Cl, 155; PO_4, 2·4 and dextrose 7·8 m-mole/l. pH was adjusted to 7·5–7·6.

RESULTS

Structural characteristics of the chorioallantoic membrane

Plate 1*a* shows the chorioallantoic membrane and the opposed uterine epithelium and submucosa from a pig foetus at 63 days of gestation. The specimen photographed had been mounted in the Ussing-type chamber and studied for approximately 6 hr before fixation in Carnoy's solution. The final measurement just before fixation showed a 30 mV transmembrane potential. The allantoic epithelium is the low cuboidal-cell layer at the bottom and is shown again at higher magnification in Pl. 1*b*. The thick stromal layer lies next above the allantoic epithelium and this in turn is surmounted by the highly folded, tall, columnar, chorionic epithelium interdigitating with the uterine endometrium. The last two epithelia are represented at higher magnification in Pl. 1*c*. A degree of separation between the chorionic and endometrial epithelia not found in fresh material has occurred in the course of study *in vitro*.

The allantois was separated from the chorion along a cleavage plane just above the line of relatively dense fibres running parallel to the allantoic epithelium.

Properties of the allantoic epithelium

Table 1 gives the rates of passage of water, sodium and chloride across the allantoic portion of the chorioallantoic membrane observed in successive periods over an interval of about 6 hr. In this experiment the membrane was tied in the form of a bag to the lower end of a polythene tube with the foetal surface inside. The composition of the fluid bathing the maternal side is shown in the footnote to the table; its osmolality was approximately equal to blood plasma. The fluid bathing the foetal surface was made by diluting it 1 to 5 with distilled water and re-equilibrating the diluted solution with 5% CO_2 and 95% O_2. The temperature was maintained at $34{\cdot}5 \pm 0{\cdot}5°$ C. The fluid bathing the maternal surface was of relatively large volume (10·0 ml.) and was constantly circulated and oxygenated by a side-arm bubble pump delivering 5% CO_2 and 95% O_2. The volume of the fluid on the foetal surface was small ($0{\cdot}8 \pm 0{\cdot}21$ ml.).

The table gives values for the net fluxes of water, sodium and chloride. Water moved from the hypotonic fluid bathing the foetal surface to the solution on the maternal side. The flux rates are consistent from period to period, uninfluenced by vasopressin and of the same order as the net fluxes of water observed in similar experiments on unstimulated frog skin (Fuhrman & Ussing, 1951) or toad bladder (Bentley, 1958). In contrast with the slow rate at which water moved out of the bag from the foetal to the maternal side, sodium and chloride passed into the sac at rates which,

while also consistent from period to period, were relatively large and indeed sufficient to reduce the initial osmotic gradient by about one half during the 40-min periods of observation. Values similar to those shown in the table were obtained in experiments in which the directions of the osmotic and chemical gradients were reversed. In all instances, the ratio of the net flux rate of sodium to that of chloride was about 1.

TABLE 1. Net fluxes of water, sodium and chloride in response to concentration gradients across the allantoic epithelium at 40 days of gestation

Period	Duration of period (min)	Net water flux (μl./cm^2. min)	Net sodium flux (μ-equiv/ cm^2.min)	Net chloride flux (μ-equiv/ cm^2.min)
1	38	0·187	—	0·240
2	49	0·117	0·176	0·163
3	39	0·135	0·223	—
4	37	0·139	0·292	0·320
5*	37	0·145	0·279	0·283
6	36	0·086	0·223	0·240
7*	38	0·120	0·284	0·296
8	36	0·113	0·315	0·312

In all periods the net flux of water was from the foetal to the maternal side, while net fluxes of sodium and chloride were from the maternal to the foetal side. Temperature 34·5° C.

Composition of the bathing fluid on the maternal side was: Na, 151; K, 5·0; Ca, 2·5; Mg, 0·2; Cl, 143; HCO_3, 16·3; PO_4, 2·5 m-equiv/l.; glucose 7·8 mM. The fluid introduced on the foetal side at the start of each period was a 1:5 dilution of that bathing the maternal surface. Hence during all periods the fluid on the maternal side at the beginning of each period had an osmolality of 278 m-osmole/kg and on the foetal side 56 m-osmole/kg. The pH on both sides was 7·5.

* During period 5 each ml. of fluid on the maternal side contained 100 m-u. and during period 7 200 m-u. of vasopressin.

No net flux of sodium, chloride or water was observed when the fluids on the two sides of the allantoic epithelium had the same composition and were isotonic with maternal blood plasma. The effect of pH on the passage of water was investigated between 7·0 and 8·4. In general, the rate was slightly higher when the pH was low. A gradient of hydrogen ion established across the epithelium was quickly dissipated.

Properties of the chorioallantoic membrane

The figures shown in Table 2 were obtained in an experiment similar to that given in Table 1, except that the full thickness of the chorioallantoic membrane from a pig at 45 days of gestation was used. As with the allantoic portion of the membrane, the net water flux in response to the osmotic gradient was small. On the other hand, the net passage of sodium from the high-concentration fluid bathing the maternal surface to the low-

concentration on the foetal side of the membrane was trifling, and the osmotic gradient across the membrane was sustained over the period of observation.

TABLE 2. Net fluxes of water and sodium in response to concentration gradients across the full thickness of the chorioallantoic membrane

Period	Duration (min)	Net water flux* (μl./cm².min)	Net sodium flux† (μ-equiv/cm².min)	Terminal osmolality on foetal side‡ (m-osmole/kg)
1	37	0·113	0·032	56·9
2	39	0·207	0·018	60·1
3	40	0·227	0·024	59·4

* From foetal to maternal side.

† From maternal to foetal side.

‡ Initial value on the maternal side 278 m-osmole/kg, and on the foetal side 56·8 m-osmole/kg.

Evidence for active sodium transport by the chorioallantoic membrane

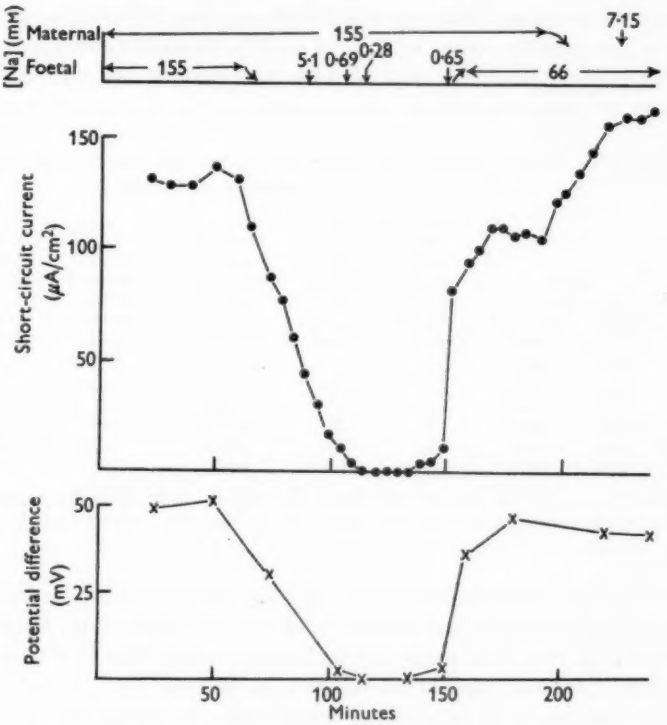
Specimens of the amnion, of the epithelium lining the allantoic cavity, of the full thickness of the chorioallantoic membrane, and of the uterine endometrium were investigated after mounting them in chambers of the design originated by Ussing (1949) to determine whether an electrical potential was developed across their surfaces. None was detected for any of these tissues except for the full thickness of the chorioallantoic membrane. Owing to its extremely friable nature it was not possible to investigate the layer of chorionic epithelium by itself.

The maternal side of the chorioallantoic membrane was always found positive to the foetal surface. Measurements made under nearly identical circumstances on specimens from pigs at 20, 33, 45 and 65 days of gestation suggested that the potential generated increased with the gestational age. Thus the maximum potential recorded at 20 days was 10 mV, but potential differences of 80 mV were observed in specimens from foetuses 45–67 days of age.

Text-figure 1 shows the effect of changing the concentration of sodium ions on the potential difference across the chorioallantoic membrane and upon the current flowing through the membrane after the spontaneous potential had been exactly balanced by the application of a counter-potential from an external source.

For the first 60 min the fluid on either side of the membrane was the same and contained 155 mM-Na. The potential difference was about 50 mV and the current about 134 μA/cm². The sodium in the fluid bathing the foetal side was then progressively replaced by choline. This was accomplished by successively withdrawing at 5 min intervals 4 ml. of fluid from

the reservoir supplying the foetal side of the chamber and replacing the withdrawn fluid with a solution identical in composition save that it contained choline instead of sodium. Since the total fluid volume in the chamber and reservoir of the foetal side of the membrane had a volume of 10 ml., each such manœuvre resulted in a reduction of the sodium concentration by approximately 40 %. The current and potential measurements shown in Text-fig. 1 are those made after allowing 4 min for re-establishment



Text-fig. 1. The effect of reducing the concentration of sodium on the foetal or maternal side of the chorioallantoic membrane on the short-circuit and potential difference across it. For the first hour of the experiment the fluids bathing the two sides of the membrane were the same and had sodium concentrations of 155 mM. At 60 min replacement of the fluid on the foetal surface with one containing choline was begun. The figures above the arrows indicate concentrations of sodium found in the foetal fluid at 90, 106 and 115 min. From this time until 151 min no further changes were made. At 151 min the sodium concentration of the foetal fluid was raised to 66 mM, and held there till the end of the experiment. At 192 min the fluid on the maternal side began to be replaced with one containing choline. At 225 min the sodium in the latter fluid had fallen to 7·15 mM, after which no further changes were made.

of a steady state. Serial measurements indicated that adjustments to the new environment were essentially complete in 2–3 min.

Potential and current fell steadily, showing in this, as in other similar experiments, a direct proportionality to the logarithm of the concentration of sodium in the fluid bathing the foetal surface of the membrane. Electrical activity became undetectable when the concentration of sodium on the foetal side reached 0·28 mM. During the next 30 min a small potential difference and short-circuit current flow reappeared. At the same time, the concentration of sodium on the foetal side was found to have risen to 0·65 mM, presumably owing to passive back diffusion of sodium from the fluid bathing the maternal side or from the stromal layer of the tissue itself. At 150 min the sodium concentration on the foetal side was raised

TABLE 3. Relations between net sodium flux and short-circuit current flow through the isolated chorioallantoic membrane

Elapsed time (min)	[Na] Foetal (mM)	[Na] Maternal (mM)	Net sodium flux: Loss, foetal side (μ-equiv/period)	Net sodium flux: Gain, maternal side (μ-equiv/period)	Rate of gain, maternal side (μ-equiv/cm².min)	Mean short-circuit current (μA)	Ion transfer predicted from short-circuit current (μ-equiv/cm².min)
0	37·8	38·4	—	—	—	—	—
90	36·1	40·4	17	15	0·053	89	0·055
153	35·3	43·9	8	25	0·128	157	0·098
225	34·4	46·3	8	17	0·074	116	0·072
300	34·1	49·0	3	18	0·074	119	0·074
365	32·8	50·6	11	10	0·049	91	0·057

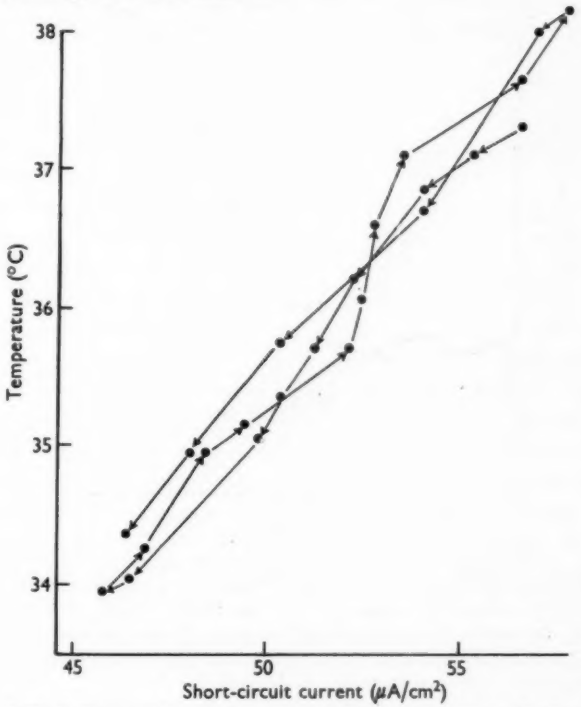
Composition of bathing solution introduced initially on both sides of membrane was: Na, 38·0; choline, 105; K, 4·7; Ca, 2·5; Mg, 0·21; Cl, 148; PO_4, 1·50 m-equiv/l.; pH, 7·5; glucose, 7·8 mM.

to 66 mM, which restored the current and the potential difference almost to the control levels. The concentration of sodium in the fluid bathing the maternal side was then reduced by choline substitution to 7·15 mM. The short-circuit current rapidly increased to more than 150 μA/cm².

Table 3 gives the results of an experiment in which the net sodium fluxes estimated from changes in the concentration of sodium on either side of the membrane were compared with the mean short-circuit current. For this purpose it was assumed that no net movement of water took place across the membrane and that the volume changes were due exclusively to the removal of samples for analysis. The validity of this assumption within 2% was established by measuring the final volumes at the end of the experiment.

It is evident from columns 2 and 3 that the sodium concentration of the fluid bathing the foetal side of the membrane fell progressively while that on the maternal side rose. The next two columns show that the net gain

of sodium on the maternal side was significantly greater in three periods than the loss from the foetal side. Inasmuch as analysis of the membrane showed it to contain much sodium, it is possible that this discrepancy, which appeared in each of the three experiments of this type, arose from the passage of sodium from the bulky, largely extracellular, stromal layer between the chorionic and allantoic epithelia, across the latter to the low-sodium fluid bathing its surface. Notwithstanding this, the rates of gain of sodium on the maternal side, shown in column 6, are in substantial agreement with the values predicted from the short-circuit current (column 7) and given in column 8.



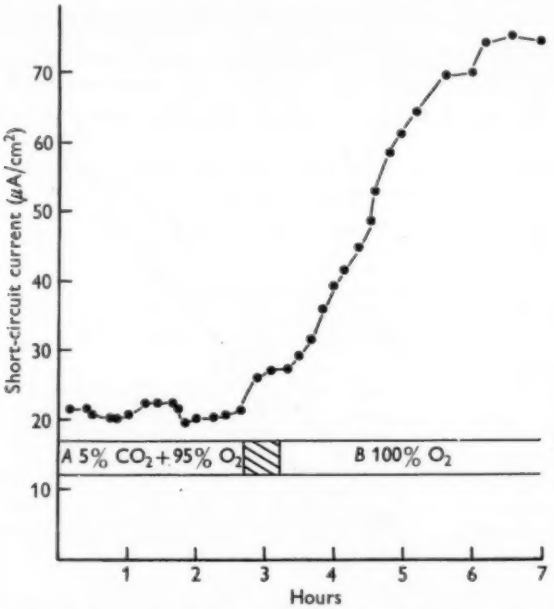
Text-fig. 2. The influence of temperature on the short-circuit current flowing through the chorioallantoic membrane. The arrows connecting the points indicate the sequence of observations.

Factors influencing the potential difference and short-circuit current flow across the chorioallantoic membrane

In Text-fig. 2 the relation between temperature and short-circuit current over a rather restricted range is shown for a membrane at 56 days gestation. Over this range the current rises in a linear fashion, increasing

6·1 %/1° C. The mean value for a series of membranes studied over an even more restricted temperature range (37–39° C) was 9·7 %/1° C. A measurable potential difference was developed at room temperature (18–24° C). The values for potential and short-circuit current fell above 41° C.

Text-figure 3 illustrates the inhibitory effect of carbon dioxide. The solution initially bathing both sides of the membrane contained 16 m-equiv bicarbonate/l., equilibrated with 5 % CO_2 and 95 % O_2. This was replaced



Text-fig. 3. The inhibitory effect of carbon dioxide on current flow through the short-circuited chorioallantoic membrane. Observations began when a solution (*A*) containing 16 m-equiv bicarbonate/l. in equilibrium at pH 7·5 with 5 % CO_2 and 95 % O_2 was in contact with both surfaces of the membrane. After 2 hr 40 min a bicarbonate-free solution (*B*) of the same pH equilibrated with 100 % O_2 was substituted on both sides of the membrane.

by one of identical pH (7·6) containing no bicarbonate and equilibrated with 100 % O_2. The increase in short-circuit current in the absence of carbonic acid is clearly evident in the figure. A corresponding rise in the potential difference across the membrane was simultaneously observed.

The largest potential differences and short-circuit current values were observed between pH 7·5 and 7·8. Reducing the pH of the solution bathing the membrane to pH 6 with hydrochloric or phosphoric acids reduced the short-circuit current to about half its initial value, and the effect was

reversible. If the same change in pH was brought about by equilibrating the solution with increasing concentrations of CO_2 in O_2 the reduction in the electrical activity was more rapid and appeared to be of greater extent but was likewise reversible.

Varying the concentration of potassium in the fluid bathing the foetal surface of the membrane from 1 to 60 mM did not appreciably affect the short-circuit current or the potential difference across the membrane. Reducing the concentration of potassium below 2 mM in the fluid bathing the maternal side greatly reduced the short-circuit current flow and the potential difference. When the potassium concentration was reduced to 0·15 mM, values approximately one third of those found in the [K] range of 2–10 mM were obtained; 2–10 mM-K appeared to be the optimal range on the maternal side. When [K] was increased to 60 mM the electrical activity fell to about half the value found in the optimal range. These effects were strikingly similar to those recently reported in the skin of the frog (Koefoed-Johnsen & Ussing, 1958).

Allantoic fluid collected from the same sac as the membrane and placed in contact only with the foetal surface strongly inhibited the electrical activity. Since this was not reduced by raising the concentration of potassium to 60 mM, that of ammonium to 30 mM, or that of calcium to 15 m-equiv/l. in artificial media bathing the foetal side of the membrane, the inhibition was probably not due to the high concentrations of these ions frequently found in the allantoic fluid. Since, as has already been mentioned, lowering the sodium in the fluid bathing the foetal surface to the levels encountered in allantoic fluid, either by dilution or by choline substitution, caused only a minor reduction in electrical activity, neither the low sodium concentration nor the osmotic gradient across the membrane was responsible for the inhibition observed when the artificial medium was replaced by the natural allantoic fluid. Allantoic fluid normally contains much CO_2. However, the inhibitory properties were retained even when the CO_2 was allowed to escape by equilibration of allantoic fluid with O_2, a process accompanied by a rise in the pH from 6·2 to 7·8.

Substitution of nitrogen for the oxygen normally bubbled through the bathing solutions caused an abrupt fall in both the potential difference and the short-circuit current. There was no suggestion that a new steady state was attained which might have been attributed to anaerobic metabolism.

Dinitrophenol caused no change in electrical activity at a concentration of 1×10^{-5} M. At a concentration of 5×10^{-5} M it caused a fall in the short-circuit current to about half of the initial value in 30 min. Washing out the drug then produced a slow but incomplete return towards normal.

The electrical activity of the membrane was uninfluenced by addition

to the fluid bathing either side of acetazoleamide, chlorothiazide or hydrochlorothiazide, to yield concentrations of 5×10^{-5} M, or by oxytocin to give concentrations of 0·5 u./ml.

DISCUSSION

It is to be regretted that in attacking the problem dealt with in the present report it was not possible to take advantage of isotope tracer techniques. These would have allowed measurements to be made of the separate unidirectional ion fluxes which contributed to the net transfer values recorded here. They would also have made it possible to attain a degree of precision superior to the best that could be achieved with the alternative techniques employed. Nevertheless, the results obtained appear to be sufficiently definite to provide a stimulus for further work on this tissue, and, even as they stand, to indicate that the chorioallantoic membrane does play an active part in determining the composition of the fluid which it envelops. The restriction of bulk water flow indicated by the data of Tables 1 and 2, and the active transfer of sodium from the contents of the allantoic sac to the maternal organism, can explain the hypotonic nature of the allantoic fluid and may also throw light upon the tendency for the accumulation in the fluid of calcium, potassium and the organic base creatinine. The transport of sodium from the chorioallantoic membrane to the maternal uterine cavity may possibly have something to do with the high concentrations of calcium in the membrane itself about the 46th day of foetal life (Economou-Mavrou & McCance, 1958). Ultimately it may appear in what way this active transport and its consequences are of importance to the development of the foetus.

The suggestion that the transport function of the chorioallantoic membrane is due to its chorionic epithelium alone cannot be advanced without reservation. It will be recalled that cornea is a tissue structurally similar to the chorioallantoic membrane, in that it consists of a thick, sparsely cellular stroma sandwiched between two epithelial layers. Herrmann & Hickman (1948*a*, *b*) have shown that there is a reciprocal metabolic relationship between corneal epithelium and stroma, in which the epithelium receives lactate from the stroma and simultaneously delivers back to the latter a co-factor necessary for its continuous glycolysis. Such a metabolic relationship could exist between neighbouring components of the chorioallantoic complex and, were this to be the case, one might well find that in the isolated state neither of the two epithelia composing the membrane was capable of active ion transport, though one or both might exhibit this function when the tissue complex was undisturbed. Granting for the moment that the sodium transport of the chorioallantois is a function of its chorionic epithelium, it is of interest to recall that Garby (1957) was unable to demonstrate a potential difference

across human amniotic membrane, while McKay, Roby, Hertig & Richardson (1955*a*, *b*) reported chemical gradients between human chorionic, amniotic and hydatidiform mole fluids, and the maternal blood plasma, which are suggestive of active ion transport. Possibly Garby's failure is explained by the care with which he discarded all specimens of amnion 'where the chorionic tissue had not been properly stripped off'.

The apparent discrepancy between the 'rapid' rates of passage of sodium and chloride ions and the 'slow' passage of water across the isolated allantoic membrane is resolved by consideration of the flux rates in comparable terms. In the circumstances of the experiment detailed in Table 1, the mean water flux was 0·130 μl./cm^2.min while that for sodium was 0·256 μ-equiv/cm^2.min. If the water flux is expressed as micromoles, the resultant figure, 7·2 μmole/cm^2.min, permits appreciation of the fact that the water movement across the membrane was, instead, relatively rapid. This is in keeping with the expectation based on data such as the figures given by Conway (1952) for free diffusion of these substances that passive water movement will be in excess of that for sodium. Thus, the allantoic epithelium, at least in its isolated state, can be characterized as a passive membrane freely permeable to water, sodium and chloride. A question which well may be raised with regard to the properties of the isolated allantoic epithelium relates to its viability in the experimental circumstances. The matter is being further investigated.

Although the volume and composition of the allantoic fluid vary progressively with the ageing of the foetus, they remain relatively constant from day to day and must be regarded as the outcome of a more or less steady state maintained by the activities of the foetal kidney and the membranes themselves. The present experiments suggest that the steady state may be maintained by a self-regulating mechanism, for the activity of the sodium pump was found to be inhibited by a rise in the concentration of hydrogen ions and by an elevation of pCO_2 in the fluid bathing the membrane without change in pH. This latter phenomenon has been noted by Ussing (1949) in amphibian membranes and explained as being due to the rapid diffusion of CO_2 across the cell membrane with lowering of the intracellular pH. Both hydrogen ion concentration and pCO_2 are high in the allantoic fluid, and it may be submitted that the activity of the sodium pump is curtailed by the products of its own activity. The specific inhibition conferred upon the sodium pump by an unidentified substance in the allantoic fluid must also be taken into account.

Further discussion would be unprofitable at this stage since there is so much more work to be done on this mammalian membrane. It would appear that the results of such study can hardly fail to advance an understanding of electrolyte stability in the foetus.

SUMMARY

1. The passage of water and several electrolytes across the isolated allantoic epithelium and the bi-epithelial chorioallantoic membrane has been studied.

2. The allantoic epithelium was found to be freely permeable to sodium, chloride and water and was electrically neutral.

3. The full thickness of the chorioallantoic membrane gave rise to an electrical potential with the maternal surface positively charged relative to the foetal side. At short-circuit, current flow appeared to be equivalent to net sodium flux across the membrane.

4. Short-circuit current and potential increased with the stage of gestation and temperature, and decreased with a fall in pH, rise in CO_2 tension and deprivation of oxygen. No short-circuit current or potential was measurable when sodium concentration on the foetal side was reduced to less than 0·3 m-equiv/l. Electrical activity was reduced in the presence of natural allantoic fluid on the foetal side and by both very low and very high potassium concentrations in the fluid bathing the maternal side. Neither electrical activity nor the passage of water across the membrane was influenced by vasopressin, acetazoleamide or chlorothiazide.

REFERENCES

ALEXANDER, D. P., NIXON, D. A., WIDDAS, W. F. & WOHLZOGEN, F. X. (1958). Gestational variations in the composition of the foetal fluids and foetal urine in the sheep. *J. Physiol.* **140**, 1–13.

BENTLEY, P. J. (1958). The effects of neurohypophyseal extracts on water transfer across the wall of the isolated urinary bladder of the toad, *Bufo marinus*. *J. Endocrin.* **17**, 201–209.

CONWAY, B. E. (1952). *Electrochemical Data*. London: Elsevier Publishing Company.

ECONOMOU-MAVROU, C. & MCCANCE, R. A. (1958). Calcium, magnesium and phosphorus in foetal tissues. *Biochem. J.* **68**, 573–580.

FUHRMAN, F. A. & USSING, H. H. (1951). A characteristic response of the isolated frog skin potential to neurohypophyseal principles and its relation to the transport of sodium and water. *J. cell. comp. Physiol.* **38**, 109–130.

GARBY, L. (1957). Studies on transfer of matter across membranes with special reference to the isolated human amniotic membrane and the exchange of amniotic fluid. *Acta physiol. scand.* **40**, Suppl. 137.

HERRMANN, H. & HICKMAN, F. H. (1948*a*). Exploratory studies on corneal metabolism. *Johns Hopk. Hosp. Bull.* **82**, 225–250.

HERRMANN, H. & HICKMAN, F. H. (1948*b*). Further studies on corneal metabolism in respect to glucose and lactic acid. *Johns Hopk. Hosp. Bull.* **82**, 260–272.

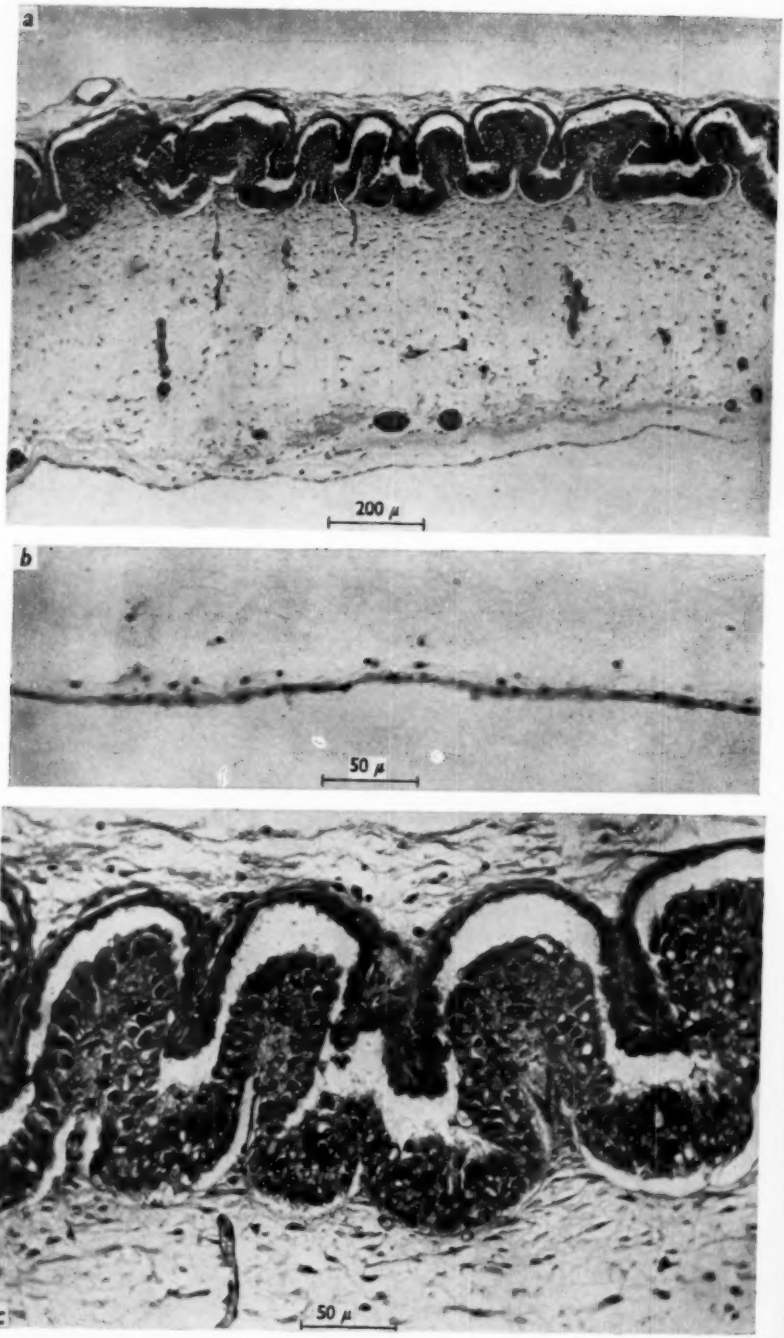
HERVEY, G. R. (1955). A method for determining the freezing points of biological fluids. *Analyst*, **80**, 284–289.

HUF, E. G., PARRISH, J. & WEATHERFORD, C. (1951). Active salt and water uptake by isolated frog skin. *Amer. J. Physiol.* **164**, 137–142.

JACQUÉ, L. (1903). De la genèse des liquides amniotique et allantoïdien. Cryoscopie et analyses chimiques. *Mém. cour. Acad. R. Belg.* **63**, 1–117.

JACQUÉ, L. (1906). De la genèse des liquides amniotique et allantoïdien. Cryoscopie et analyses chimiques. *Arch. int. Physiol.* **3**, 463–469.

KOEFOED-JOHNSEN, V. & USSING, H. H. (1958). The nature of the frog skin potential. *Acta physiol. scand.* **42**, 298–308.



J. D. CRAWFORD AND R. A. McCANCE

(*Facing p.* 471)

McCance, R. A. & Dickerson, J. W. T. (1957). The composition and origin of the foetal fluids of the pig. *J. Embryol.* **5**, 43–50.

McCance, R. A. & Widdowson, E. M. (1960). The acid–base relationships of the foetal fluids of the pig. *J. Physiol.* **151**, 484–490.

McKay, D. G., Roby, C. C., Hertig, A. T. & Richardson, M. V. (1955*a*). Studies of the function of early human trophoblast. I. Observations on the chemical composition of the fluid of hydatidiform moles. *Amer. J. Obstet. Gynec.* **69**, 722–734.

McKay, D. G., Roby, C. C., Hertig, A. T. & Richardson, M. V. (1955*b*). Studies of the function of early human trophoblast. II. Preliminary observations on certain chemical constituents of chorion and early amniotic fluid. *Amer. J. Obstet. Gynec.* **69**, 735–741.

Radde, I. C. & McCance, R. A. (1959). Glutaminase activity of the foetal membranes and kidneys of pigs. *Nature, Lond.*, **183**, 115–116.

Shales, O. & Shales, S. S. (1941). A simple and accurate method for the determination of chloride in biological fluids. *J. biol. Chem.* **140**, 879–884.

Stanier, M. W. (1960). The function of the mammalian mesonephros. *J. Physiol.* **151**, 472–478.

Ussing, H. H. (1949). The active ion transport through the isolated frog skin in the light of tracer studies. *Acta physiol. scand.* **17**, 1–37.

Widdowson, E. M. & McCance, R. A. (1958). The development of acid-base control. *Ciba Foundation Colloquia on Ageing*, **4**, 209–219.

Wislocki, G. B. (1935). On the volume of the foetal fluids in sow and cat. *Anat. Rec.* **63**, 183–192.

EXPLANATION OF PLATE

Plate 1

a. The chorioallantoic membrane and the opposed uterine epithelium from a pig foetus at 63 days of gestation fixed in Carnoy's solution after a 6 hr experiment.

b. The allantoic epithelium.

c. The chorionic epithelium with the uterine endometrium overlying it.

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THE FUNCTION OF THE MAMMALIAN MESONEPHROS

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Although a mesonephros occurs in all mammals at some stage of their development, the size and persistence of the organ varies in different species. In some, such as man and rat, the mesonephros appears early, develops few tubules, and rapidly degenerates. In others, such as sheep, pig and rabbit, the mesonephros becomes a prominent abdominal organ with many tubules, and lasts until a comparatively late stage in foetal life. In those species in which it is prominent the question arises whether the mesonephros has any physiological function, or whether its existence is merely a reflexion of mammalian phylogeny with no relation to the processes of foetal development.

It has been observed (Jacqué, 1906; Davies, 1952) that the size of the mesonephros appears to be related to that of the allantois; those species which have a large allantois have also a prominent and long-lasting mesonephros, and vice versa. The fluid produced by the mesonephros passes down the mesonephric (Wolffian) duct to the bladder and can reach the allantoic cavity through the urachus. The volume and composition of samples of allantoic fluid taken when the mesonephros was functional have been studied by many workers in several species (Jacqué, 1906; Wislocki, 1935; Davies & Routh, 1957; McCance & Dickerson, 1957); but it is not possible from such studies to make any definite statements about the function of the mesonephros. One difficulty is that the allantoic fluid may be derived from some source other than, or in addition to, the mesonephros (Wislocki, 1935; Amoroso, 1952; McCance & Widdowson, 1954); another is that the composition of the fluid may be changed after it has entered the sac by absorption or secretion through the allantoic membranes.

The mesonephric ducts open into the base of the urinary bladder, and while the mesonephros is functional and active the fluid in the bladder will be the direct product of its activity. The fluid found in the bladder was therefore collected and analysed while the mesonephros was present and active, and the results compared with those from the foetal serum on the one hand and the allantoic fluid on the other.

METHODS

Animals. Rabbits and pigs have been used. They have very different types of placenta (Amoroso, 1952), which is valuable for comparative purposes; and both have large litters. This is technically convenient when samples from the bladder or the blood serum of one foetus may not be large enough to provide sufficient material for analysis.

The observations were made at the seventeenth or eighteenth day of gestation in the rabbit, and the forty-sixth day in the pig; for at these ages the mesonephros has already been in existence for some time and is starting to degenerate, but the function of the metanephros (kidney) is not yet established (Gersh, 1937; Leeson & Baxter, 1957). In the rabbits, the doe was anaesthetized with intravenous sodium pentobarbitone, and lignocaine hydrochloride was given intramuscularly in the abdominal wall. The uterus was exposed and opened, the exo-coelomic fluid and amniotic fluids were removed, and the allantoic fluid was collected as completely as possible. The umbilical cord was tied and cut, the foetus was removed from the uterus and fixed supine on a wax block. The abdominal wall was opened by two incisions from the umbilicus to each groin. The intestines and liver were pushed aside and the bladder was exposed. It could easily be identified by the umbilical vessels running alongside its walls. The tip of the bladder at the urachus was grasped with fine forceps, the bladder was punctured and fluid was taken from it by a fine quartz tube drawn to a point at one end and fitted at the other with polythene tubing for suction. When the bladder had been emptied as completely as possible, its identity and integrity were checked by blowing air down the tube to inflate it. The chest was then opened by a mid-line incision and blood was withdrawn in the same manner from the heart. The interval between the delivery of the foetus and the taking of fluids from the bladder and heart was not less than 6 or more than 15 min. During this time the foetus was surrounded, except for its ventral surface, by warm wax (temperature 45–35° C). The samples were stored in small tubes under a layer of liquid paraffin. The dissection was performed under a binocular microscope or with head-binoculars.

In the case of pigs, the uterus and its contents were collected from sows which had just been killed at the slaughter-house. In the laboratory the uterus was opened and each foetus with its membranes was removed in turn. A sample of allantoic fluid was taken, the umbilical vessels and urachus were tied, and the foetuses were fixed in a waxed dissecting dish and treated in the same way as those of the rabbit. Samples of blood and of bladder contents were pooled to obtain sufficient material for analysis. For the majority of the observations the interval between the death of the sow and the removal of fluids from the foetus was not less than 1 or more than 5 hr. In a few cases fluids were taken from the bladder and heart of foetuses between 5 and 40 min from the death of the sow and removal of the uterus. During this time the uterus was kept warm so that its temperature remained between 37 and 34° C.

Chemical methods. In the three fluids (foetal serum, foetal bladder and allantoic fluids) the total osmolar concentrations, chloride and urea were estimated. These substances were present in measurable amounts in all three fluids.

Total osmolar concentration was measured by the cryoscopic method of Ramsay (1949). The freezing-point depression multiplied by 1000/1·86 was taken as the osmolar concentration in m-osmoles per litre.

The concentration of chloride in serum and allantoic fluid was measured by an adaptation of the method of Schales & Schales (1941) in which the sample is titrated, in the presence of the indicator diphenyl carbazone, against mercuric nitrate. Either 0·01 or 0·02 ml. samples, in duplicate, were used, according to the amount available. The chloride in the bladder fluids was measured by Wigglesworth's (1937) adaptation of the Volhard method, using 3 μl. samples in duplicate, and titrating with a horizontal burette emptying by capillarity, as described by Shaw (1955). When 3 μl. was not available, the Schales & Schales method was

adapted to samples of about 0·1 μl. by using 0·15% mercuric nitrate in a horizontal burette made of glass capillary tubing of 0·25 mm bore and calibrated by a strip of millimetre graph paper. Chloride could be recovered from standard solutions by these procedures with an error of $\pm 4\%$.

Urea was measured by a micro-adaptation of Conway's (1957) method, by using small brass Conway-units as described by Shaw & Beadle (1949) and collecting the ammonia in a hanging drop of boric acid. The error of the method was $\pm 4\%$. This method gives urea nitrogen plus the ammonia nitrogen in the sample. Of the fluids under investigation the only one which may contain appreciable amounts of ammonium is the pig's allantoic fluid (Widdowson & McCance, 1958). In any samples of this in which the ammonia had not been separately determined the mean concentration of ammonia found in twelve other samples of allantoic fluid was deducted from the observed values for urea and ammonia nitrogen and the differences taken to represent urea.

RESULTS

The results of the estimations on the three fluids are shown in Table 1. In both species there was little difference between the composition of the foetal serum and the contents of the bladder. The insignificance of the difference in the mean osmolarity and concentrations of chloride and urea between the serum and contents of the bladder is apparent from the similarity of the range of values found in all these properties in the two fluids.

As previous workers have pointed out (Davies & Routh, 1957; McCance & Dickerson, 1957) there is great variability in the composition of allantoic fluid between different litters of pigs and rabbits, and even between littermates, so average figures for allantoic fluid may be somewhat wide of the mark where individuals are concerned. The general conclusions are not likely to be invalidated by this, however, as the range of variation given in Table 1 will show. There was a great difference in mean osmolarity and concentration of chloride, between the contents of the bladder and the allantoic fluid. Although the mean concentration of urea in allantoic fluid was slightly above that in bladder fluid the difference was not significant in either species ($P = 0{\cdot}2$ for both species).

Since physiological or physical changes between the death of the sow and the removal of material from the foetus might have altered the composition of the fluids, estimations were made on material taken from a few foetuses dissected while still warm, within a few minutes of the removal of the uterus. The results of these analyses were not significantly different from those given in Table 1.

DISCUSSION

The similarity, in both pigs and rabbits, of the foetal serum and the fluid from the bladder has two possible explanations: the mesonephros may pass an unchanged plasma glomerular ultrafiltrate; or it may reabsorb from its glomerular filtrate the chloride and urea isosmotically with water, as the

TABLE 1. Composition of fluids obtained from rabbits and pigs during mesonephric function

Animal and gestation age		Total osmolar concn. (m-osmole/l.)			Chloride (m-equiv/l.)			Urea (mg/100 ml.)		
		Serum	Bladder contents	Allantoic fluid	Serum	Bladder contents	Allantoic fluid	Serum	Bladder contents	Allantoic fluid
Rabbit; 17–18 days	Mean, and no. of estimations	333 (4)	324 (4)	215 (9)	97 (3)	92 (3)	57 (9)	42 (3)	41 (3)	51 (7)
	Range	297–344	285–355	86–258	86–104	75–100	30–77	34–48	34–50	38–80
	S.D.	22	27	47	8·1	11·7	15·1	5·8	6·6	13·7
Pig; 46 days	Mean, and no. of estimations	271 (6)	265 (6)	101 (9)	96 (12)	96 (8)	24 (21)	26 (9)	27 (6)	33 (9)
	Range	243–299	223–285	54–156	88–104	83–104	5–55	18–33	18–32	21–62
	S.D.	20	17	32	4·1	6·6	16·0	6·1	3·4	12·3

Significance of differences in means (*t* test)

		Bladder contents and allantoic fluid; *P*	Bladder contents and serum; *P*
Rabbits:	Total osmolar concn.	< 0·01	0·7
	Chloride	< 0·01	0·7
	Urea	0·2	0·8
Pigs:	Total osmolar concn.	< 0·001	0·7
	Chloride	< 0·001	—
	Urea	0·2	0·3

proximal tubule of the mature kidney is thought to do. According to Patten (1927) the efferent vessel from the glomerulus of the pig's mesonephros divides to form a network of capillaries over the mesonephric tubules. This arrangement suggests that some reabsorption is occurring through the walls of these tubules. Since, however, the tubules are neither as numerous nor as convoluted as the metanephric nephrons of an adult, nor is there any segment corresponding to a loop of Henle, the amount of fluid reabsorbed is unlikely to be as great as in adults. The idea that at least a part of the water of the glomerular filtrate of the mesonephros of pigs and rabbits is reabsorbed would be consistent with the findings of Gersh (1937). This author observed an increasing concentration of injected ferrocyanide towards the distal ends of the mesonephric tubules and in the Wolffian duct. Experiments are in progress to compare the rate of glomerular filtration in the mesonephros with the passage of fluid into the bladder.

In both pigs and rabbits there was a great difference in composition between the fluid in the bladder and the allantoic fluid. If much of the allantoic fluid is the product of the mesonephros it must be considerably changed after reaching the allantoic cavity. The work of Crawford & McCance (1960) indicates that sodium may be actively removed from it by the chorioallantoic membrane. Presumably the sodium is accompanied by chloride, if no exchange of cations is taking place, and in this way the allantoic fluid is made hypotonic. The concentration of urea in the allantoic fluid, though variable, had a mean level not much greater than that in the foetal body fluids. This suggests that little if any water was being removed from the allantoic sac; or that urea was being removed at the same rate as water. However, the fact that in both species later in foetal life high concentrations of urea may be found within the allantoic sac makes the first suggestion the more attractive.

The rate of production of fluid by the mesonephros is unknown at present. The mesonephros at an early age in the foetal sheep can produce fluid at the very high rate of 1·9 ml./kg/min (Alexander, Nixon, Widdas & Wohlzogen, 1958). If the pig's mesonephros passed fluid at a similar rate (a suggestion which is not improbable, since in early foetal life the mesonephroi may comprise 20% of the body weight) the whole of the large volume of the allantoic fluid found by the forty-sixth day of gestation in the pig could have come from this source. There is nothing physically impossible in such an idea; but it is equally possible that the rate of production of mesonephric fluid is slower and that the allantoic fluid comes also from some other source, as suggested by Wislocki (1935) particularly in the first few weeks of foetal life (Amoroso, 1952; McCance & Widdowson, 1954).

The mesonephros may have an important physiological function in the foetal pig and rabbit, even if, as the present results suggest, it is doing little work in the thermodynamic sense. The passage of large volumes of fluid into the allantoic cavity may be required to keep it fully dilated and the membranes firmly apposed to the uterine wall—a mechanical function rather than a chemical one.

SUMMARY

1. The composition of serum and fluid from the bladder and allantoic sac was examined in foetuses of rabbits and pigs during the functional existence of the mesonephros. Total osmolar concentration and the concentration of chloride and urea were estimated.

2. In both species the composition of fluid from the bladder was similar to that of serum.

3. In both species the osmolar concentration and the concentration of chloride in allantoic fluid were lower than in foetal serum or bladder fluid.

Thanks are due to Professor McCance, F.R.S., for helpful criticism. The pigs and a number of the rabbits used in this work were reared at Bartlow, Cambs, and I thank Messrs Stan and Terry Cowan for taking care of the animals.

REFERENCES

Alexander, D. P., Nixon, D. A., Widdas, W. F. & Wohlzogen, F. X. (1958). Renal function in the sheep foetus. *J. Physiol.* **140**, 14–22.

Amoroso, E. C. (1952). Placentation. In *Marshall's Physiology of Reproduction*, 3rd ed. Parkes, A. S. Vol. 2, p. 127. London: Longmans, Green.

Conway, E. J. (1957). *Micro Diffusion Analysis and Volumetric Error*, 4th ed. London: Crosby Lockwood.

Crawford, J. D. & McCance, R. A. (1960). Sodium transport by the chorioallantoic membrane of the pig. *J. Physiol.* **151**, 458–471.

Davies, J. (1952). Correlated anatomical and histochemical studies on the mesonephros and placenta of the sheep. *Amer. J. Anat.* **91**, 263–290.

Davies, J. & Routh, J. I. (1957). Composition of the foetal fluids of the rabbit. *J. Embryol.* **5**, 32–39.

Gersh, I. (1937). The correlation of structure and function in the developing mesonephros and metanephros. *Contr. Embryol. Carneg. Instn*, **26**, 35–58.

Jaqué, L. (1906). De la genèse des liquides amniotique et allantoiden: cryoscopie et analyses chimiques. *Arch. int. Physiol.* **3**, 463–469.

Leeson, T. S. & Baxter, J. S. (1957). The correlation of structure and function in the mesonephros and metanephros of the rabbit. *J. Anat., Lond.*, **91**, 383–390.

McCance, R. A. & Dickerson, J. W. T. (1957). The composition and origin of the foetal fluids of the pig. *J. Embryol.* **5**, 43–50.

McCance, R. A. & Widdowson, E. M. (1954). *Cold Spr. Harb. Symp. quant. Biol.* **19**, 155–160.

Patten, B. M. (1927). *The Embryology of the Pig*, p. 192. Philadelphia: Blakiston.

Ramsay, J. A. (1949). A new method of freezing-point determinations for small quantities. *J. exp. Biol.* **26**, 57–64.

Schales, O. & Schales, S. S. (1941). A simple and accurate method for the determination of chloride in biological fluids. *J. biol. Chem.* **140**, 879–884.

SHAW, J. (1955). A simple procedure for the study of ionic regulation in small animals. *J. exp. Biol.* **32**, 57–64.

SHAW, J. & BEADLE, L. C. (1949). A simpliffed ultra-micro Kjeldahl method for the estimation of protein and total nitrogen in fluid samples of less than 1·0 μl. *J. exp. Biol.* **26**, 15–23.

WIDDOWSON, E. M. & MCCANCE, R. A. (1958). The development of acid-base control. In *Ciba Foundation Colloquia on Ageing*, **4**, 209–219. Ed. WOLSTENHOME, G. E. W. and O'CONNOR, M. London: Churchill.

WIGGLESWORTH, V. B. (1937). A simple method of volumetric analysis of small quantities of fluid: estimation of chloride in 0·3 μl. of tissue fluid. *Biochem. J.* **31**, 1719–1722.

WISLOCKI, G. B. (1935). On the volume of the foetal fluids in sow and cat. *Anat. Rec.* **63**, 183–192.

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THE FUNCTION OF THE METANEPHROS OF FOETAL RABBITS AND PIGS

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The mesonephros of foetal rabbits and pigs does not elaborate a fluid resembling urine (Stanier, 1960), analyses indicating that the fluid found in the bladder when the mesonephros is present resembles a protein-free filtrate of foetal serum. The function of the early definitive kidney (metanephros) has been investigated in a similar way; and since McCance & Dickerson (1957) and Widdowson & McCance (1958) suggested that the chorio-allantoic membrane may modify the composition of the fluid in the allantoic sac, this fluid has been compared where possible with the metanephric urine.

METHODS

Animals. Fifteen litters of rabbits at 22 days gestation and eight litters at 27 days gestation were studied (the metanephros is functional by about the 22nd day). Samples of blood from the heart, of fluid from the bladder, and, in the younger litters, of allantoic fluid, were taken from the foetuses as described by Stanier (1960); samples of blood and fluid in the bladder from litter-mates were pooled for analysis.

Eleven litters of foetal pigs of 63–67 days gestation and three litters of 90 days gestation were studied. (These ages were chosen because at 67 days the volume of allantoic fluid is about maximal; and at 90 days the allantoic fluid is fast disappearing, so that some individuals of a litter may have none and others may have 200–300 ml.) The uterus was removed within a few minutes of the sow's death, and in most cases samples of the fluids were taken from the foetuses within 60 min of the death of the sow. During this period the uterus was kept warm in an insulated container so that its temperature did not fall below 35° C. The procedure was the same as that described by Stanier (1960) except that samples of bladder fluids from individual foetuses were analysed separately.

Chemical estimations. Chloride and urea concentrations were estimated by the procedures already described (Stanier, 1960); total osmolar concentration by the cryoscopic method of Ramsay & Brown (1955); creatinine concentration by the method of Owen, Iggo, Scandrett & Stewart (1954); phosphate on 0·05 ml. samples by the method of Berenblum & Chain (1938); fructose on 0·05 ml. samples by the method of Bacon & Bell (1948).

RESULTS

The results from rabbits are shown in Table 1. It was not possible to carry out the whole programme on all three fluids from every litter. The number of figures used for calculating the mean is shown in brackets.

At 22 days gestation the three fluids did not differ greatly in their mean osmolarity. The concentration of Cl in the bladder fluids was about half that in the foetal serum, but the concentration of urea was more than three times that in the serum. At 27 days the tonicity and urea concentration of the bladder fluid were higher than at 22 days, though still below that of the urine of adult rabbits. The variability of the composition of the allantoic fluid, indicated by the high standard deviations, was in line with previous work (Davies & Routh, 1957; Dickerson & McCance, 1957).

TABLE 1. Composition of serum, bladder contents and allantoic fluid of rabbit foetuses (Mean ± S.D.; figures in brackets denote number of observations.)

Gestation age (days)	Total osmolar concentration (m-osmole/l.)			Chloride (m-equiv/l.)			Urea (mg/100 ml.)		
	Serum	Bladder contents	Allantoic fluid	Serum	Bladder contents	Allantoic fluid	Serum	Bladder contents	Allantoic fluid
22	274 (14) ± 34	234 (12) ± 63	268 (13) ± 59	98 (11) ± 5	50 (11) ± 13	44 (17) ± 20	32 (8) ± 4	114 (12) ± 10	55 (13) ± 13
27	294 (7) ± 28	299 (7) ± 52	—	93 (8) ± 14	53 (8) ± 24	—	39 (8) ± 17	173 (8) ± 80	—

TABLE 2. Composition of serum, bladder contents and allantoic fluid of 63–67-day-old pig foetuses

(Mean ± S.D.; figures in brackets denote no. of observations.)

	Serum	Bladder contents	Allantoic fluid
Total osmolar concentration (m-osmole/l.)	278 (13) ± 26	37 (11) ± 18	46 (12) ± 33
Chloride (m-equiv/l.)	94 (14) ± 3	12 (14) ± 6	18 (20) ± 7
Phosphate (mg P/100 ml.)	9·9 (9) ± 2·1	0·2 (8) ± 0·26	2·3 (6) ± 1·4
Urea (mg/100 ml.)	23 (17) ± 7	43 (17) ± 11	62 (21) ± 22
Creatinine (mg/100 ml.)	1·3 (7) ± 0·63	0·9 (7) ± 0·37	2·2 (9) ± 0·63
Fructose (mg/100 ml.)	131 (8) ± 26	61 (8) ± 33	100 (9) ± 40

TABLE 3. Composition of serum, bladder fluid and allantoic fluid of 90-day-old pig foetuses

(Mean ± S.D.; figures in brackets denote no. of observations.)

	Serum	Bladder contents	Allantoic fluid
Total osmolar concentration (m-osmole/l.)	275 (7) ± 21	26 (5) ± 16	175 (10) ± 31
Chloride (m-equiv/l.)	94 (12) ± 1·8	14 (12) ± 9·8	44 (13) ± 28
Phosphate (mg P/100 ml.)	9 (16) ± 1·8	Trace (5)	Trace (8)
Urea (mg/100 ml.)	29 (8) ± 6	69 (9) ± 6·5	114 (12) ± 48
Creatinine (mg/100 ml.)	0·45 (6) ± 0·2	1·9 (5) ± 0·6	17 (8) ± 2·3
Fructose (mg/100 ml.)	99 (9) ± 17	44 (9) ± 22	216 (9) ± 92

The results from pigs are given in Tables 2 and 3. At both ages the fluid taken from the bladder contained very little chloride and phosphate, but a higher concentration of urea than foetal serum. At both ages the concentration of fructose found in the vesicular fluid was about half that found in the serum. At 67 days gestation the allantoic fluid had a very

low level of chloride, but a higher urea concentration than that of the bladder fluids. At 90 days allantoic fluid still present was more concentrated.

DISCUSSION

Although chloride and phosphate were the only ions estimated in this investigation, their relationship to sodium in serum and allantoic fluid is known from previous work (McCance & Dickerson, 1957). Sodium is known to move through most animal cells by active transport (Ussing, 1949), the movement of chloride being secondary. However, the phrase 'chloride reabsorption' has been used in this paper in preference to 'sodium chloride reabsorption' because sodium levels were not measured. Unless urea is secreted through the wall of the kidney tubule into the lumen (a process which has not been proved in any mammalian species) an increased urea concentration in the urine as compared with the blood must be interpreted qualitatively as removal of water; but as the amount (if any) of urea lost by back diffusion in the foetal kidney is not known, the actual amount of water reabsorbed cannot at present be calculated.

The present results showed that by the twenty-second day of gestation the kidney of the rabbit foetus was forming a fluid resembling urine. The concentration of urea in the bladder fluid was about three times that in the serum; and this suggested that at least two-thirds of the water of the glomerular filtrate was reabsorbed. Simultaneously, chloride must have been reabsorbed to an even greater extent than water; the concentration of this ion in urine was only half that in the serum, whereas its isosmotic reabsorption with water would have resulted in equal concentrations in serum and urine. By the twenty-seventh day (4 days before term) the concentration of urea in the fluid of the bladder was 4 or 5 times that in the serum; this indicated that removal of water from the glomerular filtrate was more extensive than at 22 days.

The pig foetuses showed an interesting difference from the rabbits. Chloride and phosphate appeared to be reabsorbed in the kidney tubules very efficiently, even in the younger of the two sets of foetuses; but judging by the degree to which urea and creatinine had been concentrated, little water was reabsorbed in either age group, though somewhat more at 90 than at 67 days.

The finding of low tonicity and low chloride and phosphate levels in the urine is in line with the observations of McCance & Widdowson (1953) on human foetal urine. However, both osmolarity and chloride concentration of pig foetal urine at the stages described in this study are even lower than the concentration in human foetal urine at any stage.

In the allantoic fluid of the 67-day pig foetus the mean concentrations of all the organic constituents were about twice as high as in the fluid in

the bladder. This suggested that water was leaving the allantoic sac more quickly than urea and the other organic substances could diffuse out of it. The removal of water presumably continued very quickly during the next few weeks. Consequently, at 90 days many foetuses had no allantoic fluid left, and such allantoic fluids as remained showed a high concentration of urea, and also a higher chloride level than at 67 days.

The kidney of the 22-day-old rabbit foetus reabsorbed both chloride and water from the glomerular filtrate; its water reabsorption (as judged by urea concentration in the bladder) was more effective by 27 days. The kidney of the 67-day pig foetus removed chloride very efficiently from the glomerular filtrate; and at this stage, water may have been reabsorbed in the allantoic sac, as suggested by the high concentration of urea found there. By 90 days gestation the kidney of the pig foetuses appeared to reabsorb both chloride and water efficiently, and allantoic fluid was disappearing.

SUMMARY

1. Blood serum, allantoic fluid and bladder fluid have been taken from foetal rabbits and pigs at two stages of gestation after the development of the metanephros. The concentration of chloride and urea and the tonicity of these three fluids have been measured.

2. In rabbit foetuses at both stages the concentration of urea in bladder fluid was higher, and that of chloride lower, than in the serum.

3. In pig foetuses at both stages the concentration of chloride in the bladder fluid was much lower than that in the serum; and the concentration of urea was higher.

4. Allantoic fluids of both species varied considerably in composition but differed always in some respects from fluids taken from the bladder. Such allantoic fluid as was still present at the later stage of gestation in the pig contained a high concentration of urea, and a higher mean tonicity and mean chloride level than at 67 days.

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REFERENCES

BACON, J. S. D. & BELL, D. J. (1948). Fructose and glucose in the blood of the foetal sheep. *Biochem. J.* **42**, 397–405.

BERENBLUM, I. & CHAIN, E. (1938). An improved method for the colorimetric determination of phosphate. *Biochem. J.* **32**, 295–298.

DAVIES, J. & ROUTH, J. I. (1957). Composition of the foetal fluids of the rabbit. *J. Embryol.* **5**, 32–39.

DICKERSON, J. W. T. & MCCANCE, R. A. (1957). The composition and origin of the allantoic fluid in the rabbit. *J. Embryol.* **5**, 40–42.

McCance, R. A. & Dickerson, J. W. T. (1957). The composition and origin of the foetal fluids of the pig. *J. Embryol.* **5**, 43–50.

McCance, R. A. & Widdowson, E. M. (1953). Renal function before birth. *Proc. Roy. Soc.* B, **141**, 488–497.

Owen, J. A., Iggo, B., Scandrett, F. J. & Stewart, C. P. (1954). The determination of creatinine in plasma or serum and in urine: a critical examination. *Biochem. J.* **58**, 426–437.

Ramsay, J. A. & Brown, R. H. J. (1955). Simplified apparatus and procedure for freezing-point determinations upon small volumes of fluid. *J. sci. Instrum.* **32**, 372–375.

Stanier, M. W. (1960). The function of the mammalian mesonephros. *J. Physiol.* **151**, 472–478.

Ussing, H. H. (1949). The active ion transport through the isolated frog skin in the light of tracer studies. *Acta physiol. scand.* **17**, 1–37.

Widdowson, E. M. & McCance, R. A. (1958). The development of acid–base control. In *Ciba Foundation Colloquia on Ageing*, ed. Wolstenholme, G. E. W. and O'Connor, M., pp. 209–219. London: Churchill.

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THE ACID–BASE RELATIONSHIPS OF THE FOETAL FLUIDS OF THE PIG

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The foetal fluids of the pig, sheep, cow and rabbit have frequently been studied, but the investigations have tended to be sporadic rather than systematic. McCance & Dickerson (1957) gave a short account of how the composition of these fluids in the pig changed during the first 67 days of gestation, and discussed the origin of the fluids in the light of their own results and previous work on this species and also on the rabbit (Dickerson & McCance, 1957). Since that time further work has been carried out on the sheep (Alexander, Nixon, Widdas & Wohlzogen, 1958) and the pig (Economou-Mavrou & McCance, 1958), but some of the observations on the pig have only been reported in a preliminary way at a Ciba symposium (Widdowson & McCance, 1958), and some are now published (Crawford & McCance, 1960; Stanier, 1960; McCance & Stanier, 1960). The object of this short communication is to extend the previous studies to a later age, and to consider specifically the acid–base relationships, particularly in the allantoic fluid; the results will be discussed in relation to theories about the origin of the allantoic fluid.

METHODS

Twenty-five cross-bred gilts or sows were used to provide material for this investigation. (Large White, Essex, Wessex and Landrace crosses were included.) The sows were killed at the slaughterhouse at 22, 44–46, 66–67 and 89–90 days gestation; the uterus was removed at once and brought to the laboratory as quickly as possible for further dissection. The volumes of allantoic and amniotic fluid in all the sacs were measured and samples taken for analysis. Pieces of the various foetal membranes and uterine wall, as far as possible with no blood vessels in them, were taken for carbonic anhydrase determination and placed immediately at $-10°$ C. The foetuses were weighed, the stomachs, kidneys and mesonephros, when present, were removed and stored at $-10°$ C for the estimation of carbonic anhydrase.

The chemical methods used for the analysis of the fluids were those described by Dickerson & McCance (1957) and Economou-Mavrou & McCance (1958). Inorganic sulphate was determined by the method recommended by Hawk, Oser & Summerson (1954). Since Widdowson & McCance (1958) had previously found that the allantoic fluid of the pig was acid when first removed from the sac, and that the acidity was largely due to carbonic acid, the titratable acid was measured by withdrawing 2–4 ml. of fluid from the sac into a 10 ml.

syringe containing a drop of mercury. A measured volume (2–5 ml.) of 0·01 NaOH was placed in a small beaker and a few drops of phenol red added. Some of this NaOH was drawn up into the syringe containing the fluid, the syringe being rotated so that the mercury facilitated mixing, until the colour indicated a pH of 7·4. The excess NaOH in the beaker was then titrated against 0·01 N-HCl. The pH was determined with B.D.H. capillators, with the minimum exposure to air. Ammonia was estimated either by distilling in a micro-Kjeldahl apparatus at pH 8·0, or by the method described by Conway (1957). The results obtained by the two methods agreed very closely. A van Slyke apparatus was used for the determination of CO_2, the fluid being transferred directly into the apparatus from the syringe in which it was collected.

Carbonic anhydrase in the fluids, membranes and organs was estimated by the semi-quantitative method of Brinkmann (1933) as described by Robinson (1950). The fluids were used directly, but weighed samples of the tissues were first ground with an equal weight of a saline-phosphate buffer (248 ml. M/15-NaCl + 12 ml. M/15 phosphate buffer, pH 7·4), the suspension centrifuged and the supernatant fluid used for the determination of enzyme activity (Lutwak-Mann, 1955). It was sometimes necessary to dilute the fluid with saline-phosphate buffer if the material was particularly rich in the enzyme. One enzyme unit (e.u.) was defined as the amount which would reduce the reaction time from 65–75 sec to 25–30 sec (Keilin & Mann, 1940).

RESULTS

Table 1 shows the number of litters and fluids which have been investigated at 22, 46, 67 and 90 days after insemination; the volumes of allantoic fluid are given next, and then the information which has been obtained about its composition—pH, titratable acid, ammonia, sodium, potassium, calcium, magnesium, chloride, inorganic phosphorus, inorganic sulphur, citric acid and CO_2. The last three lines summarize the new figures for urea, creatinine and total osmolal material.

The volumes followed the recognized trends up to 67 days. From the figure of 5 ml. previously given for 20 days (McCance & Dickerson, 1957) it would appear that the volume is increasing very quickly just at that time. At 90 days the fluids in the allantoic sac are shrinking rapidly, so that some sacs contained none and others a volume of more than a litre.

At 22 days the pH was 7·0, and may have been higher before that time, but by 46 days it had fallen to 6·0 and remained at about that level until the observations ceased at 90 days. The titratable acid was considerable, though not high by urine standards, owing to the dilute nature of the fluid and the relatively small amount of buffer substances in it. There were, however, appreciable amounts of ammonia, which had neutralized roughly 25% of the H ions secreted into the fluid, i.e. the ammonia accounted for about this proportion of the titratable acid + ammonia. The acidity in these fluids was largely due to carbonic acid and the pH rose rapidly on exposure to air. The findings for the sodium, chloride and potassium at 22, 46 and 67 days corroborate previous work, but the rise in the concentration of potassium, calcium, chloride and inorganic sulphate at 90 days is a new observation and worth comment. There was also a

major rise in the concentration of creatinine at this time, but not of urea. The amount of osmolal material doubled itself between 67 and 90 days, largely in consequence of these increases.

TABLE 1. Composition of allantoic fluid

(Mean ± S.D.)

Stage of gestation. Days ...	22	46	67	90
No. of fluids analysed	39	61	31	18
No. of litters involved	4	7	4	3
Volume (ml.) Mean	31	62	328	—
Range	16–48	20–291	83–1000	0–1260
	Composition of fluid			
pH	7·0 ± 0·05	6·0 ± 0·25	6·1 ± 0·29	5·8 ± 0·80
Titratable acid*	4·7 ± 0·95	14·7 ± 7·7	8·5 ± 3·4	11·0 ± 3·2
Ammonia*	0·6 ± 0·19	4·5 ± 2·3	2·2 ± 1·0	3·6 ± 1·8
Na*	60·5 ± 11·8	9·9 ± 5·1	7·9 ± 4·2	4·1 ± 1·8
K*	9·5 ± 2·0	4·5 ± 1·2	12·6 ± 3·3	76·7 ± 25·9
Ca*	2·9 ± 0·7†	19·2 ± 4·0	14·5 ± 5·4	34·9 ± 8·3
Mg*	1·5 ± 1·1†	0·9 ± 0·5†	0·45 ± 0·25	5·0 ± 2·2
Cl*	36·3 ± 6·1	28·4 ± 13·5	19·4 ± 8·1	75·8 ± 6·8
Inorganic P mM*	2·8 ± 0·6†	1·9 ± 0·6†	1·1 ± 0·3	1·0 ± 0·4
Inorganic S*	—	—	3·0 ± 0·5	12·4 ± 1·9
Citric acid*	—	1·6 ± 0·5	1·2 ± 0·8	1·6 ± 0·3
CO_2*	33·3 ± 4·5	10·6 ± 1·0	7·4 ± 1·4	9·4 ± 2·4
Urea (mg/100 ml.)	33·6 ± 4·8	36·8 ± 2·7	84·2 ± 18·6	106 ± 39·8
Creatinine (mg/100 ml.)	2·2 ± 1·1	3·0 ± 1·1	2·8 ± 0·9	16·5 ± 2·2
Total osmolal concentration (m-osmole/l.)	149 ± 16	104 ± 24	100 ± 18	237 ± 27

* m-equiv/l. † Figures from Economou-Mavrou & McCance (1958).

TABLE 2. Composition of amniotic fluid and of fluid removed from the foetal stomachs at 67 and 90 days gestation

	Amniotic fluid		Fluid removed from foetal stomachs	
Stage of gestation. Days ...	67	90	67	90
Volume (ml.) Average	163	155	—	—
Range	110–305	47–320	—	—
pH	7·2	7·2	6·5	6·8
Na*	117	111	126	120
K*	10·0	8·4	12·0	3·8
Cl*	94	91	109	108
Total osmolal concentration (m-osmole/l.)	273	273	—	—

* m-equiv/l.

Table 2 shows the information which has been obtained about the composition of the amniotic fluid at 67 and 90 days gestation. Figures for the earlier stages of foetal life were given by McCance & Dickerson (1957). The chemical 'structure' of the fluid changed relatively little throughout these stages of its functional life and was quite different from that of the allantoic fluid. This applied particularly to the pH, and the concentration of sodium, chloride and osmolal material.

Table 2 also shows the average composition of fluid removed from foetal

stomachs at 67 and 90 days gestation. It resembled that of amniotic fluid but for the more acid pH. There was a small excess of inorganic cations in both fluids.

The pH of these fluids, particularly of the allantoic fluid, and the concentration of sodium and chloride in it, led to an examination of various internal organs, and of the membranes and fluids for carbonic anhydrase, and the results are given in Table 3. The erythrocytes at 46 days contained about 8000 e.u. of enzyme per gram and were the most active tissue examined, but the mesonephros was highly active at 46 days and the true kidney as active or more so by 67 days. The chorioallantoic membrane

TABLE 3. Carbonic anhydrase in foetal membranes and organs

(Results expressed as enzyme units per gram fresh tissue; average and range are given.)

Stage of gestation. Days ...	22	46	67	90
Chorioallantoic membrane	150 (80–250)	250 (200–275)	—	200 (180–250)
Chorion alone	—	320 (300–360)	320 (300–330)	—
Allantoic membrane alone	—	170 (100–300)	150 (110–200)	100
Allantoic 'tips'	—	380 (190–600)	300	1200
Amniotic membrane	—	150 (60–200)	70	80 (50–100)
Uterine wall	—	150 (100–200)	300	200
Allantoic fluid	—	50 (20–80)	50 (50–50)	50 (50–50)
Foetal Mesonephros	—	1400	—	—
Foetal Metanephros	—	1100	2400	2400
Foetal Stomach	—	130	600	600

was also active and, as found by Lutwak-Mann (1955), the activity was greater in the chorionic than in the allantoic layers, while the 'tips', which contained more solids and less water (Economou-Mavrou & McCance, 1958), were the most active part of the whole area of the membrane. Measurable activity was found even in the fluid itself and the activity was just as high in the clear supernatant fluid as in the deposit obtained after centrifugation. The wall of the stomach, and of the uterus, in agreement with Lutwak-Mann (1955), also displayed some activity, and the amniotic membrane was quite active too. No activity was detected in the amniotic fluid, Wharton's jelly or in human amniochorion.

DISCUSSION

Physiological significance of the pH measurements

In the normal course of events it took some time to remove the uterus from the pig at the slaughter house, transfer it to the laboratory and dissect out the foetuses, and the following steps were taken to exclude

the possibility that the low pH and the high concentrations of carbonic acid were due to anaerobic foetal metabolism after the death of the sow. All these experiments were made at 46 days gestation. (*a*) A small incision was made in the abdomen of one sow immediately after death, one horn of the uterus removed and samples of the allantoic fluids in it obtained at once. The other horn was removed at leisure and brought to the laboratory. The pH of the first fluid, measured 15 min after death, was around 6·0, and it was not appreciably lower (5·8) in the last fluid to be taken 2·5 hr later. (*b*) Fluids were removed from the allantoic sacs of anaesthetized sows during life and found to have a pH 6·0–6·1, and titratable acidity of 8–14 m-equiv/l.

Carbonic anhydrase and the pH of the allantoic fluid

Carbonic anhydrase is found at sites in the body where active secretion involving a change of pH is in progress, and its presence in the kidney, pancreas and stomach are good examples. It is, however, not confined to these sites and its presence and activity in the reproductive tract of a number of animals was described by Lutwak-Mann (1955). In this investigation it has been found in the mesonephros, kidney, stomach and chorio-allantoic membrane, where the secretion of H ions might be expected, or has been shown to take place, and also in other parts of the foetus such as the amniotic membrane, where its role in the functional life of the tissue has not yet been made clear.

The ammonia in the allantoic fluid

The way in which the ammonia formed by the kidney finds its way into the urine is still a matter of investigation; and the same is true of the gills of fish (Krogh, 1939). If, as suggested by Robinson (1954), the ammonia is formed in the renal cells by the normal processes of metabolism, and is trapped when it diffuses into an acid but not into a neutral or alkaline fluid in the tubules, a similar theory would explain its presence in the acid allantoic fluid, whether it is present in the fluid entering the sac or accumulates in the fluid after its arrival there. There is glutaminase activity at all the necessary sites (Radde & McCance, 1959).

The composition, origin and function of the fluids

The figures given in Table 1 and those previously obtained (McCance & Dickerson, 1957) show that during the functional life of the allantoic sac the fluid it contains becomes progressively more hypotonic until about the 67th day, when the volume is at its maximum (Pomeroy, 1956). The hypotonicity is largely due to the removal of sodium and chloride. By the 90th day the fluid is beginning to be absorbed and disappear, and the

increase in concentration of potassium and calcium have become considerable and are accompanied by a corresponding increase in chloride and inorganic sulphate. If the cations are added together and set against the sum of the various anions measured in the fluids, there is a small excess of cations which were probably bound by organic acids, but the two figures agree reasonably well, and together account for a satisfactory amount of the total osmolal concentrations as determined from the freezing points. There are, therefore, no major discrepancies in the ionic balance sheet.

The anatomical relationships and connexions indicate, as has often been suggested, that the sac must act as a receptacle for the fluid secreted by the mesonephros and early metanephros, but the work of Stanier (1960), Crawford & McCance (1960) and McCance & Stanier (1960) indicates that the fluids passing into the sac from the bladder must be greatly modified during their storage there, probably by the chorioallantoic membranes. It still remains to be discovered just how the fluid is created in the early stages of foetal life and how its composition is altered by the active reabsorption of sodium, accompanied by chloride to some extent, and possibly with some simultaneous ionic exchange of sodium for hydrogen and other radicles. Further discussion now would be premature, but the next few years should see progress made in this field of foetal physiology.

SUMMARY

1. The allantoic and other fluids, together with certain tissues and membranes of the foetal pig, have been examined at 22, 46, 67 and 90 days gestation.

2. By 46 days the pH of the allantoic fluid was about 6·0. The fluid contained ammonium ions and the acidity was largely due to carbonic acid.

3. The chorioallantoic membrane, the amniotic membrane, the foetal mesonephros, metanephros and stomach all contained active carbonic anhydrase. There was measurable activity in the allantoic fluid.

4. The concentrations of potassium, calcium, chloride, inorganic sulphate and creatinine in the allantoic fluid rose to a considerable height at 90 days, when the functional life of the sac was nearly over, but the concentration of sodium continued to fall.

A number of people have given us their help at various stages of this investigation, notably Drs C. Polge, M. W. Stanier and J. W. T. Dickerson, Mr T. Cowen, Miss R. Lowings and Mrs J. Briggs.

REFERENCES

ALEXANDER, D. P., NIXON, D. A., WIDDAS, W. F. & WOHLZOGEN, F. X. (1958). Gestational variations in the composition of the foetal fluids and foetal urine in the sheep. *J. Physiol.* **140**, 1–13.

BRINKMANN, R. (1933). The occurrence of carbonic anhydrase in lower marine animals. *J. Physiol.* **80**, 171–173.

CONWAY, E. J. (1957). *Microdiffusion Analyses and Volumetric Error*, 4th ed., p. 98. London: Crosby Lockwood.

CRAWFORD, J. D. & MCCANCE, R. A. (1960). Sodium transport by the chorioallantoic membrane of the pig. *J. Physiol.* **151**, 458–471.

DICKERSON, J. W. T. & MCCANCE, R. A. (1957). The composition and origin of the allantoic fluid in the rabbit. *J. Embryol.* **5**, 40–42.

ECONOMOU-MAVROU, C. & MCCANCE, R. A. (1958). Calcium, magnesium and phosphorus in foetal tissues. *Biochem. J.* **68**, 573–580.

HAWK, P. B., OSER, B. L. & SUMMERSON, W. H. (1954). *Practical Physiological Chemistry*, 13th ed., p. 949. London: Churchill.

KEILIN, D. & MANN, T. (1940). Carbonic anhydrase. Purification and nature of the enzyme. *Biochem. J.* **34**, 1163–1176.

KROGH, A. (1939). *Osmotic Regulation in Aquatic Animals*, p. 127. Cambridge University Press.

LUTWAK-MANN, C. (1955). Carbonic anhydrase in the female reproductive tract. Occurrence, distribution and hormonal dependence. *J. Endocrin.* **13**, 26–38.

MCCANCE, R. A. & DICKERSON, J. W. T. (1957). The composition and origin of the foetal fluids of the pig. *J. Embryol.* **5**, 43–50.

MCCANCE, R. A. & STANIER, M. W. (1960). The function of the metanephros of foetal rabbits and pigs. *J. Physiol.* **151**, 479–483.

POMEROY, R. W. (1956). *Reproduction in the sow with special reference to infertility and neonatal mortality*. Ph.D. thesis, University of Cambridge.

RADDE, I. C. & MCCANCE, R. A. (1959). Glutaminase activity of the foetal membranes and kidneys of pigs. *Nature, Lond.*, **183**, 115–116.

ROBINSON, J. R. (1950). Urinary excretion of carbonic anhydrase. *J. clin. Path.* **3**, 142–145.

ROBINSON, J. R. (1954). *Reflections on Renal Function*, p. 110. Oxford: Blackwell.

STANIER, M. W. (1960). The function of the mammalian mesonephros. *J. Physiol.* **151**, 472–478.

WIDDOWSON, E. M. & MCCANCE, R. A. (1958). The development of acid–base control. In *Ciba Foundation Colloquia on Ageing*, **4**, 209–219, ed. WOLSTENHOLME, G. E. W. and O'CONNOR, M. London: Churchill.

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QUANTITATIVE DESCRIPTION OF SODIUM CURRENTS IN MYELINATED NERVE FIBRES OF *XENOPUS LAEVIS*

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Earlier experiments (Dodge & Frankenhaeuser, 1959) on the myelinated nerve fibre demonstrated that the initial ionic current is caused by sodium ions moving passively down their electro-chemical gradient. These studies, carried out by using the voltage clamp technique, also showed that (1) the sodium-carrying mechanism can be described in terms of the sodium permeability (P_{Na}) of the membrane, when the permeability is calculated from the constant-field equation for a simple membrane, (2) the sodium permeability depends on the membrane potential and (3) the permeability changes with finite speed in response to a change in membrane potential. Further studies were concerned with the effect of conditioning polarization on the available sodium permeability (Frankenhaeuser, 1959).

The present investigation is concerned with the time course of the change in sodium permeability. The recorded current consists of a capacitive current, an unspecific leak current, a sodium current and a delayed ionic current. Before further analysis can be carried out, sodium current has to be separated out from the other currents. The capacitive current and the leak current can be estimated from records taken with anodal pulses and subtracted from the recorded total currents. At times later than the peak of the initial current the delayed ionic current begins. In order to separate the sodium current from the delayed ionic current it is necessary either that the delayed current should be determined independently, as can be done in low external sodium concentrations (Hodgkin & Huxley, 1952*a*; Dodge & Frankenhaeuser, 1959), or that the time course of the sodium permeability change should be determined from the instantaneous current–voltage relations by means of records taken at sudden repolarizations to a value of membrane potential that is near the equilibrium potential for the delayed current (Hodgkin & Huxley, 1952*b*; Dodge & Frankenhaeuser, 1958). The latter method, although more elaborate, was preferred in the present investigation, since experiments with choline solutions showed some diminution of the delayed ionic current that would have given an apparent reversal of sodium current at later times for large cathodal

pulses. This effect suggests that choline chloride is a not altogether inert replacement for sodium. The time course of the change of P_{Na} was hence determined at various times during a number of different polarizations and the rate constants α_m, β_m, α_h and β_h which determined P_{Na} were calculated. The formal assumptions made for this calculation are very closely related to those made for the squid fibre voltage-clamp data (Hodgkin & Huxley, 1952*d*). Empirical equations were then fitted to the experimental data. This set of equations ought to describe the sodium currents for any time course of potential change within the potential range investigated. The total ionic currents cannot, however, be described before the delayed currents have been treated quantitatively. The delayed currents will be analysed in a subsequent paper.

METHODS

The membrane potential of a node in a large myelinated nerve fibre from the sciatic nerve of *Xenopus laevis* was changed in rectangular steps. The membrane potential was controlled by a feed-back amplifier system (Dodge & Frankenhaeuser, 1958, 1959) and the current calibration was made as previously described (Dodge & Frankenhaeuser, 1959).

The Ringer's solution had the following composition (mM): NaCl 112·0, KCl 2·5, $CaCl_2$ 2·0, $NaHCO_3$ 2·5.

Procedure. Several clamp runs were taken in each experiment. (*a*) The pulse amplitude was varied (e.g. Dodge & Frankenhaeuser, 1959, Fig. 1). The sodium equilibrium potential (V_{Na}), the curve relating peak sodium current to membrane potential (I_{Na}–V_m curve), and the curve relating peak sodium permeability to membrane potential (P_{Na}–V_m curve) were obtained from these records (e.g. Dodge & Frankenhaeuser, 1959, Figs. 2 and 3). (*b*) The membrane potential was changed in two steps. The first, conditioning, step polarized the membrane to various values, while the second, test, step took V to a value close to that potential at which sodium current was at its maximum (as a rule to $V = +57$ mV). The curve for steady state inactivation against membrane potential (h_∞–V_m curve) was obtained from these records (e.g. Hodgkin & Huxley, 1952*c*, Fig. 5; Frankenhaeuser, 1959, Fig. 3). (*c*) Double pulses were applied, by means of which conditioning polarizations of various amplitudes and various durations preceded a test step, making $V = +57$ mV (at which potential the initial inward current is maximal). The time course of inactivation at various membrane potentials was determined from this run (e.g. Hodgkin & Huxley, 1952*c*, Fig. 3; Dodge & Frankenhaeuser, 1958, Fig. 8). (*d*) Pulses of various amplitudes were applied and interrupted at various times after the onset. The repolarization gave a tail of sodium current (e.g. Hodgkin & Huxley, 1952*b*, Fig. 10; Dodge & Frankenhaeuser, 1958, Fig. 9) from which the time course of the P_{Na} change was determined. (*e*) Anodal pulses were applied. Capacitive currents and leak currents were estimated from these records. (*f*) A number of runs were taken for an analysis of the delayed currents. These will be treated in a subsequent paper.

The order in which runs were taken was varied from experiment to experiment. All the runs were not taken in each individual experiment. Systematic runs were taken on about 40 axons. As a rule interest was concentrated in order to establish single details rather than to give a complete quantitative analysis, and quite a number of experiments were made at temperatures other than room temperature. Towards the end of the experimental series a few experiments were made so as to give a relatively complete over-all picture at room temperature (20° C). These experiments were by no means the most stable ones, and scatter

between measured points was less in many other experiments. However, it was decided to concentrate for the quantitative description in the present paper on the data obtained from these four axons.

Axons were numbered in order to allow comparison of data between figures and tables in the two preceding papers and in this one. The numbers were not in chronological order of experiments.

Nomenclature. Potentials are given as inside potential minus outside potential, and outward current as positive. E is used for absolute values of potentials. V is used for potentials relative to the resting potential, thus $V = E - E_r$.

FORMAL TREATMENT AND ASSUMPTIONS

A number of formal assumptions were made in order to make it possible to derive the empirical equations that describe the sodium currents in the myelinated nerve. These assumptions were intended to be as closely related as possible to those made by Hodgkin & Huxley (1952*d*) for the squid fibre voltage-clamp data.

In a previous paper (Dodge & Frankenhaeuser, 1959) it was shown that the sodium-carrying properties of the membrane at the time of the peak of the initial ionic current could be satisfactorily described by the sodium permeability (P_{Na}) of the membrane with the constant-field equation. The assumptions required for this conclusion are those required for the derivation of the constant-field equation, and the assumption that P_{Na} of the membrane changes with finite speed. A further step is to assume that Na concentration changes rapidly within the membrane, and reaches a steady state so quickly that the transients can be neglected. Considering the dimensions of the membrane this is a plausible approximation, and the observations have been consistent with the idea; but it must be emphasized that the resolution of the technique is severely limited at very short times ($< 0{\cdot}02$ msec) after the voltage step is applied. The sodium current (I_{Na}) would thus at any instant and at any membrane potential be determined by the P_{Na} of the membrane according to the constant-field equation. Thus

$$I_{Na} = P_{Na} \frac{F^2 E}{RT} [Na]_0 \frac{\exp\{(E - E_{Na}) F/RT\} - 1}{\exp\{EF/RT\} - 1}. \quad (1)$$

Sodium current is thus linearly related to P_{Na} at any one voltage-clamp step (since E is held constant during step). Changes in P_{Na} with time can thus be determined from measurements of I_{Na}, and it is clear from the earlier measurements that P_{Na} changes continuously with time and with membrane potential.

For cathodal steps, sodium current rises with an S-shaped time course and is transient, whereas for a repolarization sodium current decays with an exponential time course. In order to obtain a formal equation which accounts for this, it was assumed by analogy with the treatment of the

squid fibre data (Hodgkin & Huxley, 1952*d*) that P_{Na} is a power function of a first-order equation. The time course of the sodium current was compared with a family of curves with the form

$$(1-\exp\{-t/\tau_1\})^a \times \exp\{-t/\tau_2\}.$$

This revealed that $a = 2$ gave a somewhat better fit with the experimental points than $a = 3$, which was used for the squid fibre data. The delay in rise of the sodium current in the *Xenopus* fibre was thus found to be somewhat smaller than in the squid fibre.

Thus

$$P_{Na} = \bar{P}_{Na}\, m^2 h, \tag{2}$$

$$\frac{dm}{dt} = \alpha_m (1-m) - \beta_m m, \tag{3}$$

$$\frac{dh}{dt} = \alpha_h (1-h) - \beta_h h; \tag{4}$$

where $\bar{P}_{Na}$ is a constant with dimensions of permeability and has the numerical value that P_{Na} would take if $m = 1$ and $h = 1$; m and h are dimensionless variables which can vary between 0 and 1; α_m and β_m, and α_h and β_h, are rate constants which vary with membrane potential but not with time, and have dimensions of (time)$^{-1}$.

During a voltage-clamp step, V changes suddenly and then remains constant. α_m, β_m, α_h and β_h then change instantly to a new value that is appropriate to the new value of the membrane potential. The solutions for equations (3) and (4), when $m = m_0$ and $h = h_0$ at $t = 0$, are then

$$m = m_\infty - (m_\infty - m_0) \exp(-t/\tau_m), \tag{5}$$

$$h = h_\infty - (h_\infty - h_0) \exp(-t/\tau_h), \tag{6}$$

where

$$m_\infty = \alpha_m/(\alpha_m + \beta_m), \tag{7}$$

$$\tau_m = 1/(\alpha_m + \beta_m), \tag{8}$$

$$h_\infty = \alpha_h/(\alpha_h + \beta_h), \tag{9}$$

and

$$\tau_h = 1/(\alpha_h + \beta_h). \tag{10}$$

Combining equations (7) and (8),

$$\alpha_m = \frac{m_\infty}{\tau_m} \quad \text{and} \quad \beta_m = (1-m_\infty)/\tau_m. \tag{11), (12}$$

Combining equations (9) and (10),

$$\alpha_h = \frac{h_\infty}{\tau_h} \quad \text{and} \quad \beta_h = (1-h_\infty)/\tau_h. \tag{13), (14}$$

As a rule the membrane potential was at its resting value before the pulse was applied, and at this potential m was negligibly small compared

with its maximal value during the pulse. m_0 could therefore be neglected when the pulse was larger than about $+30$ mV, and h_∞ was nearly zero for such pulses. In this case the solution of equation (2) is:

$$P_{Na} = P'_{Na}\{1-\exp(-t/\tau_m)\}^2\{\exp(-t/\tau_h)\}, \quad (15)$$

where $P'_{Na} = \bar{P}_{Na}\, m_\infty^2\, h_0$, that is to say P'_{Na} has the value which the P_{Na} would attain if inactivation were to remain at its resting value.

METHOD OF ANALYSIS

Voltage-clamp runs (*a–f*) were taken as described on p. 492, and the following data were extracted from the records and tabulated: *A*. The peak $I_{Na}-V$ relation was measured from runs (*a*) and (*e*). *B*. The sodium equilibrium potential V_{Na} or E_{Na} was determined from (*a*) and (*e*). *C*. The peak $P_{Na}-V$ relation was calculated from equation (1) by using the data in *A* and *B*. *D*. The steady state $h_\infty-V$ relation was measured from runs (*b*) and (*e*). *E*. The time constant for the change of h, τ_h, at various values of V between about -50 and $+40$ mV, was measured from runs (*c*) and (*e*). *F*. The time course of the change of P_{Na} at various potentials was determined from runs (*d*) and (*e*). *G*. The data obtained in *F* were plotted on double-log. paper and compared with a plot of a family of solutions of equation (15) with various values of τ_m/τ_h. *H*. P'_{Na} was obtained from *C* and *G*. *I*. $\bar{P}_{Na}$ was obtained from *C*, *D* and *G*. *K*. The $m_\infty-V$ relation was obtained from *H*, m_∞ being the square root of the ratio of P'_{Na} to the asymptote value of P'_{Na} at large cathodal pulses. *L*. τ_m and τ_h were obtained from *G*. *M*. The values of α_m, β_m, α_h and β_h were then calculated from equations (11), (12), (13) and (14).

RESULTS

The experimental values for the four rate constants are plotted against V in Figs. 1–4, m_∞ is plotted against V in Fig. 5, and the values of P_{Na} and V_{Na} are given in Table 1. The asymptote value of P'_{Na} at large pulses was determined by drawing a smooth line through a number of single determinations of P'_{Na} at large cathodal pulses, and the value of maximally available I_{Na} was determined from a smooth line drawn through the peak currents with a number of large anodal conditioning polarizations. Otherwise the calculation of the individual values in Fig. 1–5 was based on 'single point observations'.

The next step was to fit empirical equations to the experimental values of the rate constants. It seemed clear that α_m, β_m and β_h could reasonably well be fitted with a common curve for all the individual fibres, while the values for α_h fell on separate curves for the individual fibres.

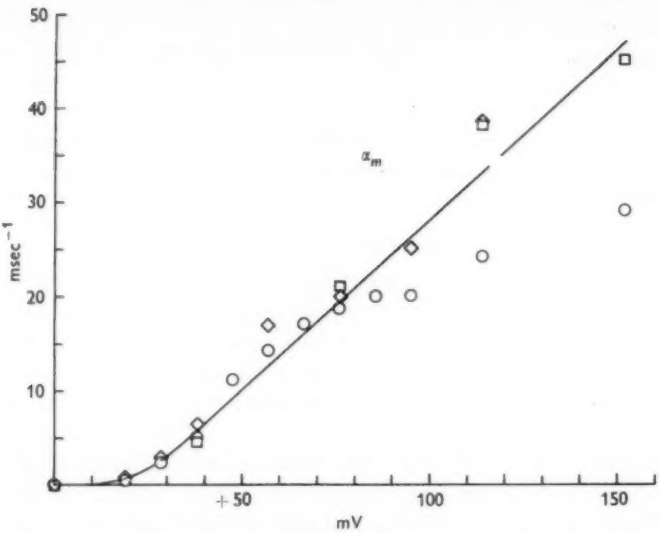

Fig. 1. Rate constant α_m of sodium permeability plotted against V. Continuous line is the solution of equation (16). 20° C. Axon 10 ◇, 11 □, 12 ○.

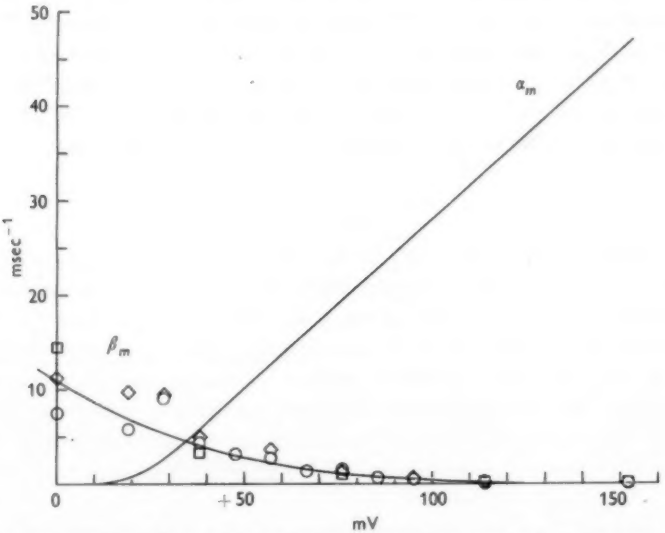

Fig. 2. Rate constant β_m of sodium permeability plotted against V. The continuous line for β_m drawn as solution of equation (17). The curve for α_m drawn in for comparison. 20° C. Axon 10 ◇, 11 □, 12 ○.

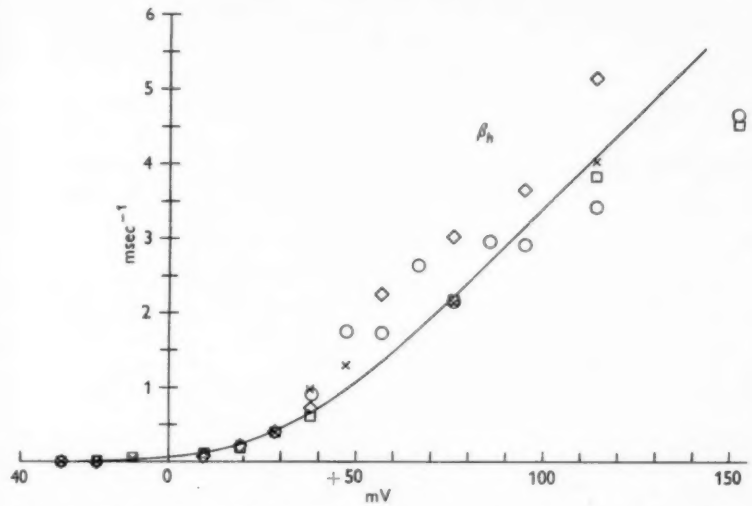

Fig. 3. Rate constant β_h of inactivation plotted against V. The continuous line for β_h drawn as solution of equation (19). 20° C. Axon 9 ×, 10 ◇, 11 □, 12 ○.

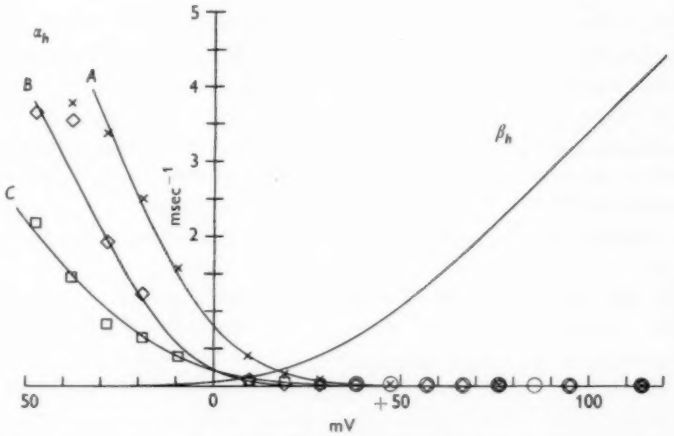

Fig. 4. Rate constant α_h of inactivation plotted against V. Continuous lines for α_h drawn as solutions of equation (18). The curve for β_h drawn in for comparison. 20° C. Axon 9 ×, 10 ◇, 11 □, 12 ○.

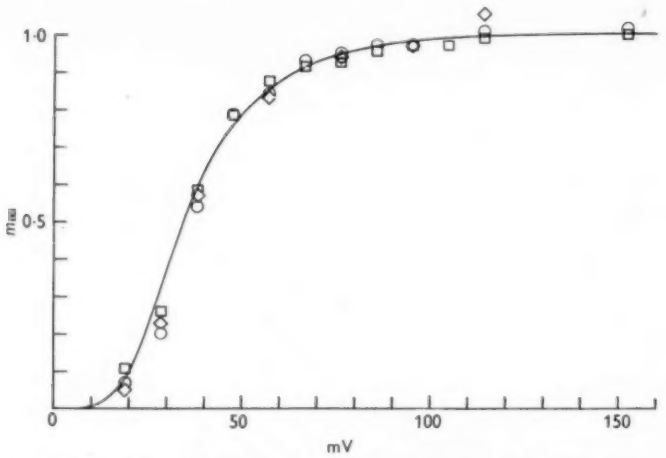

Fig. 5. m_∞ plotted against V. Continuous line drawn as solution of equation (7). 20° C. Axon 10 ◇, 11 □, 12 ○.

TABLE 1

Axon	P_{Na} (cm/sec; $\times 10^{-3}$)	P'_{Na} (cm/sec; $\times 10^{-3}$)	$\bar{P}_{Na}$ (cm/sec; $\times 10^{-3}$)	V_{Na} (mV)	E_{Na} (mV)	$g_L = \frac{I_L}{E - E_R}$ (m-mho/cm²)
9	4·0	6·0	6·7	+126·2	+56·2	25·4
10	2·9	5·1	6·6	+120·0	+50·0	26·5
11	2·5	3·6	6·1	+125·0	+55·0	28·2
12	4·6	8·2	12·4	+120·2	+50·2	41·0
Mean	3·5	5·7	8·0	+122·9	+52·9	30·3

E_R is taken as −70 mV. Some of the values deviate from values given for corresponding axons in Table 1 of Frankenhaeuser (1959). Measurements were made on different runs, and no corrections have been made for deterioration of the fibres during the experiment.

The continuous lines in Figs. 1–5 are drawn according to the equations:

$$\alpha_m = 0{\cdot}36\,(V-22)\Big/\left\{1-\exp\left(\frac{22-V}{3}\right)\right\}, \tag{16}$$

$$\beta_m = 0{\cdot}4\,(13-V)\Big/\left\{1-\exp\left(\frac{V-13}{20}\right)\right\}, \tag{17}$$

$$\alpha_h = a(b-V)\Big/\left\{1-\exp\left(\frac{V-b}{c}\right)\right\}, \tag{18}$$

where the values of a, b and c were

Axon	Curve	a	b (mV)	c (mV)
9	*A*	0·14	− 5	+ 8
10	*B*	0·10	−10	+ 6
11	*C*	0·07	−20	+10

$$\beta_h = 0{\cdot}05\,(V-32)\Big/\left\{1-\exp\left(\frac{32-V}{10}\right)\right\} \tag{19}$$

and
$$m_\infty = \alpha_m/(\alpha_m+\beta_m), \tag{7}$$

where α_m and β_m have the values given by equations (16) and (17) in msec^{-1} at 20° C.

The equation (1) in the preceding paper (Frankenhaeuser, 1959) for the $h_\infty - V$ relation is an approximation for

$$h_\infty = \alpha_h/(\alpha_h+\beta_h), \tag{9}$$

where α_h and β_h have the values given by equations (17) and (18).

The general form of equation which was chosen for all the rate constants is the same as that used by Hodgkin & Huxley (1952*d*) for α_m (note the difference in nomenclature). β_h did not, as in the squid fibre data, reach a limiting value at high values of V. This was tested on other fibres to +180 mV. The membrane breaks down at slightly higher potentials, so it was impossible to check it at still higher values. α_h and β_m could not be fitted with simple exponential curves. This was especially the case when β_m was measured at −57 mV. (These experiments were made at other temperatures and are therefore not included in the figures of this paper.)

DISCUSSION

The rate constants that govern the sodium-carrying mechanism in the myelinated nerve are described by equations (16)–(19) as graded smooth functions of the membrane potential. There was a fair amount of scatter between the points in Figs. 1–4. This scatter was certainly due to small errors in the measurements and not due to a step-wise turn on (or off) of the permeability of certain regions of the membrane in an all-or-none manner, since the membrane currents were smoothly graded with time and also with membrane potential at very small (2 mV) changes in potential steps. The following question then arises: do these equations describe the sodium permeability changes in the single 'sodium channel' or do they describe the average behaviour of a great number of single channels? The possibilities of observing the behaviour of a single channel seems more favourable with isolated nodes than with squid fibres, since the surface area of the node is very small compared to the area on which measurements are made on the squid fibre. So far it can be said that the resolution of the technique was such that quantal responses of a size of 1% of the maximal inward currents could have been detected, but were not in fact seen. Therefore, the only conclusion that can be drawn is: *either* there are very many sites (> 100) that turn on their sodium permeability either in a graded or in a quantal fashion, *or* the number of sites is

small, but in this case each single site must show a graded change in permeability. The 'all-or-none responses' reported by Tasaki & Bak (1958) are of quite a different order of size and can hardly be caused by the response of single sites.

In the present treatment, P_{Na} was described as being proportional to m^2 (equation 2) because this gave a better fit with the experimental data than m^3. The sodium currents were slightly more delayed if the fibre was polarized anodally before applying the test pulse. The third power could conceivably be correct if some permeability were already turned on at the resting potential. The experiments did not give any indications in this direction. Therefore m^2 was used, and not m^3 as for the squid fibre data (Hodgkin & Huxley, 1952*d*).

It was shown that m_∞, α_m, β_m and β_h were nearly the same in all the fibres, while h_∞ and α_h varied from fibre to fibre. The threshold (i.e. the membrane potential at which a regenerative action potential develops) of the fibres did not vary much between individual fibres. Threshold potential mainly depends on α_m and β_m, if $\bar{P}_{Na}$ and non-specific permeability are the same for all the fibres. The response to linearly rising currents and the accommodation are again known to vary largely. This variation seems at present to be accounted for by the variation in α_h. (These conclusions are drawn with some reservation because a complete quantitative treatment cannot be made until the delayed currents have been analysed quantitatively.) Accommodation is commonly held to be closely associated with calcium, and the external calcium concentration is known to change the accommodation. Calcium, however, changes all the rate constants in squid axons (Frankenhaeuser & Hodgkin, 1957), while only α_h was found to vary from fibre to fibre. It seems therefore likely either that calcium has very little to do with the variation in accommodation in normal ionic environments, or that, if calcium plays an important part, then something else must compensate for the effect of calcium on α_m, β_m and β_h.

The empirical equations describing the relation of sodium permeability to membrane potential and to time are consistent with the idea that charged particles moving in an electric field govern the permeability changes. Further, the sodium permeability of the membrane appears to be independent of sodium concentration and independent of membrane current, and the permeability changes are smoothly graded with potential and with time. Thus these observations indicate that while there are some differences between the sodium permeability changes of the squid giant fibre and the frog myelinated nerve fibre nevertheless the similarities are very striking.

SUMMARY

1. The sodium currents (I_{Na}) were analysed in voltage-clamp experiments on the myelinated nerve fibre of *Xenopus laevis*. The analysis was made in terms of the sodium permeability (P_{Na}) of the membrane, and equations were fitted to the experimental data.

2. These empirical equations (2) to (19), describe how P_{Na} depended on membrane potential and on time.

3. The analysis indicates striking similarities in the sodium transport system in the frog fibre and in the squid fibre, although a number of differences were noted.

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REFERENCES

DODGE, F. A. & FRANKENHAEUSER, B. (1958). Membrane currents in isolated frog nerve fibre under voltage clamp conditions. *J. Physiol.* **143**, 76–90.

DODGE, F. A. & FRANKENHAEUSER, B. (1959). Sodium currents in the myelinated nerve fibre of *Xenopus laevis* investigated with the voltage clamp technique. *J. Physiol.* **148**, 188–200.

FRANKENHAEUSER, B. (1959). Steady state inactivation of sodium permeability in myelinated nerve fibres of *Xenopus laevis*. *J. Physiol.* **148**, 671–676.

FRANKENHAEUSER, B. & HODGKIN, A. L. (1957). The action of calcium on the electrical properties of squid axons. *J. Physiol.* **137**, 218–244.

HODGKIN, A. L. & HUXLEY, A. F. (1952*a*). Currents carried by sodium and potassium ions through the membrane of the giant axon of *Loligo*. *J. Physiol.* **116**, 449–472.

HODGKIN, A. L. & HUXLEY, A. F. (1952*b*). The components of membrane conductance in the giant axon of *Loligo*. *J. Physiol.* **116**, 473–496.

HODGKIN, A. L. & HUXLEY, A. F. (1952*c*). The dual effect of membrane potential on sodium conductance in the giant axon of *Loligo*. *J. Physiol.* **116**, 497–506.

HODGKIN, A. L. & HUXLEY, A. F. (1952*d*). A quantitative description of membrane current and its application to conduction and excitation in nerve. *J. Physiol.* **117**, 500–544.

TASAKI, I. & BAK, A. F. (1958). Current–voltage relations of single nodes of Ranvier as examined by voltage-clamp technique. *J. Neurophysiol.* **21**, 124–137.

J. Physiol. (1960), **151**, *pp.* 502–517
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SCRATCHING MOVEMENTS EVOKED BY DRUGS APPLIED TO THE UPPER CERVICAL CORD

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The following experiments give a description of scratching movements elicited, not reflexly, but by the action of drugs when applied topically to the upper cervical cord. The experiments are the outcome of an incidental observation made in cats anaesthetized with pentobarbitone sodium, in which the dye bromophenol blue was perfused through the cerebral ventricles, and its penetration into the brain tissue was studied (Feldberg & Fleischhauer, 1960). In these experiments perfusion was effected from a cannulated lateral ventricle to the cannulated aqueduct, and as long as the dye did not enter the subarachnoid space no central effects were observed. However, in a few experiments in which the dye leaked out into the subarachnoid space, either along the outside of the aqueductal cannula or because of rupture of the thin roof of the posterior part of the third ventricle, strong myoclonic movements of the hind legs occurred. They were regularly produced when the dye was perfused from the lateral ventricle to the cisterna.

These findings suggested that bromophenol blue had a central excitatory action when present in the subarachnoid space but not when passing through the cerebral ventricles or the aqueduct. When trying to locate the site of action of bromophenol blue by topical application to different parts of the cerebrum, cerebellum, brain stem and spinal cord, it was found that the myoclonic movements could be elicited only from a restricted area of the dorsal surface of the upper cervical cord and that they showed a striking resemblance to those of a normal scratch reflex. Further, similar scratching movements were obtained from the same area with tubocurarine, which is known to produce muscular effects also when perfused from the lateral ventricle to the aqueduct, that is, without passing into the subarachnoid space (Feldberg & Malcolm, 1959).

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METHODS

The experiments were performed on anaesthetized cats. Usually, and if not otherwise stated, anaesthesia was effected by intraperitoneal injection of pentobarbitone sodium (35 mg/kg); additional pentobarbitone was injected during the course of a prolonged experiment. In a few experiments intravenous chloralose (80 mg/kg) was used. The trachea was cannulated and, with the cat lying on its belly, the head was fixed to the ear bars and mouthpiece of a head holder similar to the Horsley Clark stereotaxic instrument. The muscles covering the atlanto-occipital membrane and the epistropheus were dissected away, and the arcs of the atlas and epistropheus were removed, care being taken not to open the venous sinuses on each side. The margin of the supra-occipital bone at the border of the foramen magnum was nibbled away, exposing the lower part of the vermis. The dura was then opened in the mid line and folded back, so as to expose the dorsal surface of the lower part of the medulla oblongata and of the upper cervical cord, for the topical application of either bromophenol blue or tubocurarine solutions.

When the solutions were applied to one side of the cord only, the applicator was a cotton wick threaded through a syringe needle in such a way that about 1 cm of the wick protruded from the tip of the needle, which was kept in a vertical position. The protruding end of the cotton wick was placed on the selected region and a continuous slow flow of solution was maintained by keeping the upper end of the needle filled. When applied to both sides of the cord, a small swab of cotton wool soaked in the solution was placed on the selected region. In order to ensure a good penetration of the topically applied solutions into the tissue of the cord, it was essential that the surface of the cord was not allowed to dry or to become covered by a film of clotted blood.

The sodium salt of bromophenol blue and the tubocurarine were dissolved in artificial c.s.f. The composition of this solution has been described by Merlis (1940). The bromophenol blue was obtained commercially (British Drug Houses) as the acid. It is a powder insoluble in water. The water-soluble sodium salt was prepared by grinding the powder in a mortar with equimolecular amounts of N/10-NaOH; i.e. about 6 ml./400 mg powder, as described previously (Feldberg & Fleischhauer, 1960). The sodium salt has a blue-violet colour.

The tibialis anterior muscles were chosen when it was intended to record the muscular effects from the flexor muscles of the hind legs. With the legs in a flexed position, both tibiae were fixed on a Brown–Schuster myograph stand and the freed tendons of the tibialis muscles were attached to myographs. The contractions were recorded on a smoked drum.

RESULTS

In cats under pentobarbitone sodium anaesthesia strong muscular effects resembling the pattern of the scratch reflex are obtained when a 0·2 or 1 % solution of bromophenol blue or a 0·1 % solution of tubocurarine is applied to the dorsal surface of the spinal cord at the level of C1. In Fig. 1 the shaded area is the region from which this pattern of muscular activity is obtained. When the solutions are applied a little lower than this region, or to the exposed dorsal surface of the lower thoracic cord, no muscular effects are obtained.

When the bromophenol blue or the tubocurarine is applied to one side of the region from which the muscular effects are elicited, the scratching movements occur on that side only. They are sometimes preceded by fasciculation of the muscles in the thigh and in the lower part of the back.

This fasciculation is predominantly ipsilateral and occurs 2–3 min after the topical application. The scratching movements begin a little later in the ipsilateral hind leg with a strong sustained flexion at the hip, and the foot is brought forward along the side of the body. From this position strong myoclonic bursts of movement of the leg are set off by rhythmic alternate flexion and extension at the hip, knee and ankle. There is plantar flexion

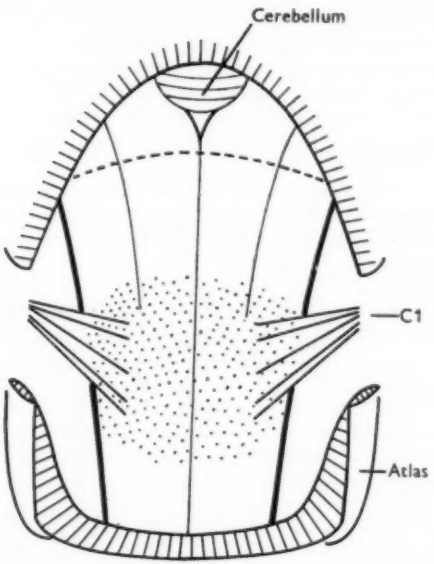

Fig. 1. Diagram of exposed dorsal surface of upper cervical cord of the cat. The stippled area is the region from which scratching movements are obtained with bromophenol blue and tubocurarine. The lower margin of the occipital bone and the arc of the atlas, except its lower rim, have been nibbled away. C1, dorsal roots of first cervical nerve. The interrupted line indicates high transverse section which does not abolish the effect.

of the digits, so that the paw becomes claw-shaped, and the claws are partly extruded. These bursts of scratching movements occur at irregular intervals and are often associated with a steady extension and forward thrust of the ipsilateral foreleg, which is also slowly adducted so that it crosses the mid line. The movement of the foreleg is that which a cat performs when wiping its face. Ipsilateral twitching of the ears and of the facial muscles occurs regularly during these scratching bursts and sometimes also in the intervals. During the scratching movements there is an increase in extensor tone in the contralateral hind leg, which sometimes becomes rigidly extended.

In most experiments the head was fixed in a head holder; thus any head

and body movements which might accompany the scratching movements were masked or prevented. However, in those experiments in which the head was taken out of the holder it became evident that the scratching movements were associated with the postural attitude taken up by a cat when scratching itself behind the ear. The vertebral column was bent with its concavity directed towards the scratching limb, the head was turned to the same side and then rotated so that the claws of the scratching foot touched the skin behind the ear.

When the bromophenol blue or the tubocurarine is applied to both sides of the region from which the muscular effects are elicited, the scratching movements occur in both hind legs, but at different times. They start in one hind limb and when they come to an end they may be immediately followed by similar scratching movements in the opposite hind limb, and so the scratching movements continue alternating from one hind limb to the other, or there may be periods of rest in both hind limbs between these alternating scratching movements. When the scratching movements alternate quickly from one hind limb to the other, and the head is fixed in a head holder, the slow steady extension and adduction of the corresponding forelegs does not keep pace with the quick alternation, with the result that the forelegs cross. When this quick alternation occurs with the head taken out of the head holder, the bending of the spine and turning and rotation of the head also occurs alternately from one side to the other. Apart from these movements there is a general increase in tone of the body musculature instead of the usual relaxation of muscle tone present in pentobarbitone sodium anaesthesia.

In two cats which were decerebrated at mid-collicular level in pentobarbitone sodium anaesthesia, the topical application of bromophenol blue to the region of the spinal cord shown by the stippled area in Fig. 1 produced the same muscular effects as in the non-decerebrate cats.

Penetration of bromophenol blue into the tissue of the cervical cord

In several experiments, in which the topical application of 0·2 % bromophenol blue with a cotton swab to both sides of the dorsal surface of the cervical spinal cord at the level of C1 had led to the typical bursts of scratching movements, cross-sections of the cord were made at this level at the end of the experiment and examined with the naked eye for penetration of the dye into the tissue of the cervical cord. It was found that within half an hour the dye had penetrated about 1–2 mm into the nervous tissue; with longer periods of topical application there was little increase in the depth of penetration but the staining became more intense. Usually the zona gelatinosa Rolandi stood out within the stained area as a more intensely stained region, as illustrated in the diagram of Fig. 2.

Myographic recordings from the anterior tibialis muscles of cats in pentobarbitone anaesthesia

Bromophenol blue. When the dye is applied to the region of the cervical cord shown by the shaded area in Fig. 1, it causes contractions of the tibialis muscles, but not when it is applied to lower regions of the cervical, or to the dorsal surface of the thoracic cord. The concentration of dye used was usually 0·2% but sometimes 1%.

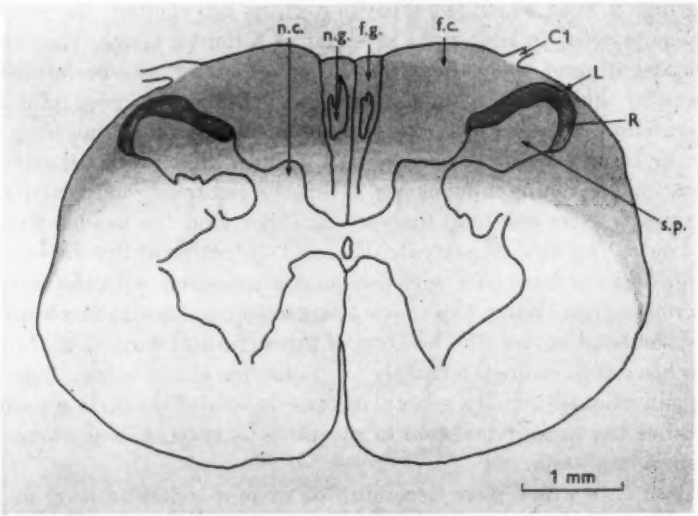

Fig. 2. Cross-section of cat's spinal cord at level of C1. Penetration of bromophenol blue 30 min after topical application to the dorsal surface shown by darkened area. Zona gelatinosa Rolandi (R) stands out, being more intensely stained. n.c. and f.c., nucleus and fasciculus cuneatus; n.g. and f.g., nucleus and fasciculus gracilis; C1, entrance of first cervical dorsal root; L, area of Lissauer; s.p., stratum spongiosum internum. Terminology from Winkler & Potter (1914).

When the dye is applied to one side of the region shown by the shaded area of Fig. 1, the effect occurs in the ipsilateral tibialis muscle only; it usually begins after a latency of 3–10 min with a few irregular and comparatively weak contractions followed by a burst of strong rhythmic contractions. Similar bursts recur at irregular intervals and usually follow a typical pattern. They begin with a sustained contraction, a strong tension is built up step-wise, then it suddenly gives way, relaxation becomes either complete or partial and the muscle is thrown into vigorous rhythmic activity. The duration of a burst as well as the strength and frequency of

its rhythmic contractions vary in different experiments and also in the course of the same experiment.

Figure 3 illustrates two typical bursts. The upper record shows a burst in which there is complete relaxation of the initial sustained contraction and again after each subsequent contraction of the rhythmic activity. This was the more usual occurrence. The record further illustrates the irregular

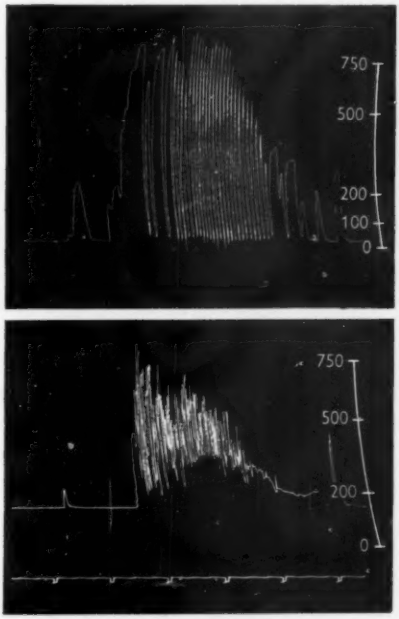

Fig. 3. Records from anterior tibialis muscle of two cats under pentobarbitone sodium anaesthesia. Two types of bursts of scratching movements obtained on topical application of 0·2% bromophenol blue to the dorsal surface of the upper cervical cord. Upper record: scratching movements at a frequency of 1·3/sec after initial strong contraction; complete relaxation between beats. Lower record: frequency of the scratching movements 3·3/sec; incomplete relaxation between beats. On the right, tension in grams. Time marker, 10 sec.

reduced and more sustained contractions at the end of the burst. The lower record is from a different experiment. The initial sustained contraction of the burst relaxes slightly only before the onset of the rhythmic activity and relaxation between successive rhythmic beats is also incomplete. The frequency of beats is about 1·3/sec in the burst of the upper, and about 3·3/sec in that of the lower record. In both records the initial strong sustained contraction of the burst is preceded by a smaller sustained contraction. Sometimes several such small sustained contractions occur a few seconds before the onset of the actual burst.

Before each burst the tibialis muscle is either relaxed or undergoes irregular contractions, which are weaker and occur at lower frequencies than the bursts, so that these stand out clearly. Sometimes, however, bursts are no longer distinguishable from the almost continuous activity which varies in intensity from time to time. In the same experiment the pattern of activity may change from discrete bursts separated by periods of rest to an almost continuous activity.

During the bursts of the anterior tibialis muscle other muscles of the same limb undergo similar rhythmic contractions, but as long as the dye does not diffuse across the mid line no contractions occur in the opposite hind limb and its tibialis muscle remains relaxed.

In two experiments in which the topical application of bromophenol blue had produced almost continuous activity of the tibialis muscle this activity ceased as a spontaneous emptying of the bladder occurred, and began again after micturition.

When the bromophenol blue is applied to both sides of the spinal cord, at the region shown by the shaded area in Fig. 1, the tibialis muscles become active in both limbs but they contract alternately. The onset of a burst in one tibialis muscle causes cessation of contraction in the other and when this muscle again begins to contract, the activity in the former ceases. This alternation, which is illustrated in Fig. 4, shows certain variations. A burst of activity may start in one tibialis muscle immediately or a short time after the activity in the other has come to an end. Or, when the bursts are of short duration and occur infrequently, one, two or three may occur in the same tibialis muscle during a relatively long period of rest in the other. At other times a burst in one tibialis muscle may start during the final weak contractions of a subsiding burst in the other; or again a burst may start in one whilst the other relaxes from a strong sustained contraction, which may have been the initial phase of a burst cut short by the onset of the burst in the muscle of the other side. This is seen in Fig. 4. The onset of the last burst in the left tibialis muscle coincides with the relaxation from a strong sustained contraction of the right muscle. There is not only an alternation of activity in both tibialis muscles but the pattern of activity may be different too; discrete infrequent bursts may be evoked in one and long periods of almost continuous activity in the other.

The fact that the onset of activity in one tibialis muscle is the cause of cessation of activity in the other is sometimes strikingly illustrated in experiments in which the dye is applied first to one and then to the other side. When the first application has produced almost continuous activity in the one muscle and the dye is then applied to the other side, this activity ceases abruptly with the onset of activity in the other muscle.

When the dye is removed and the surface of the cervical cord is frequently rinsed with artificial c.s.f., the muscular activity in the tibialis muscle subsides and ceases within 40–60 min, but on re-application of the dye it returns.

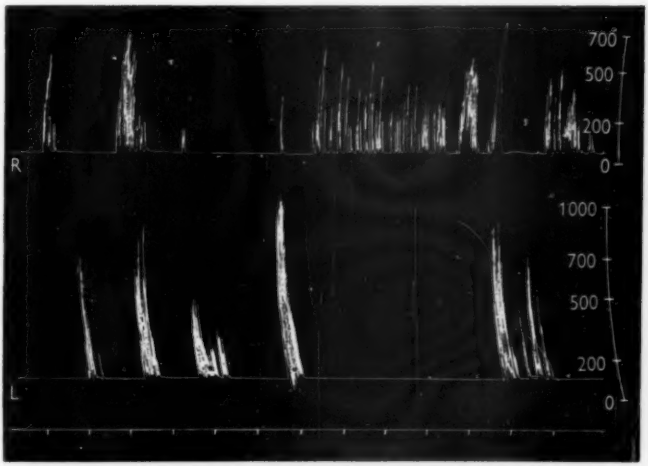

Fig. 4. Record of alternating activity in right (R) and left (L) anterior tibialis muscle of a cat under pentobarbitone sodium anaesthesia a few minutes after topical application of 0·2 % bromophenol blue to both sides of the exposed dorsal surface of the upper cervical cord. On the right, tension in grams. Time marker, 10 sec.

Tubocurarine. The topical application of tubocurarine, in a 0·1 % solution, to the dorsal surface of the spinal cord has the same effect as bromophenol blue. Applied to the region shown by the shaded area in Fig. 1 it causes contractions of the tibialis muscles, but applied at a lower level of the cervical cord it is ineffective. Applied to one side the effect occurs on the ipsilateral tibialis muscle, applied to both sides both tibialis muscles become alternately active. Between the application of the tubocurarine and the onset of contractions there is a latency of 5–15 min, which is on the average a little longer than after bromophenol blue, and on removal of the tubocurarine the activity subsides more quickly than after removal of the bromophenol blue. The pattern of activity of the tibialis muscle is the same as after bromophenol blue. There are the discrete characteristic bursts of rhythmic contractions, but the activity may change to an almost continuous one. Some of these results are illustrated in Figs. 5 and 6.

Figure 5 shows the beginning of muscular activity in the left anterior tibialis muscle a few minutes after the topical application of tubocurarine

to the same side of the cervical cord. A few short-lasting contractions are followed by discrete bursts, the last one being taken with a quicker speed of the drum to show the frequency of the rhythmic contractions, which is about 2/sec.

Figure 6 shows that after rinsing the surface of the spinal cord with artificial c.s.f. following removal of the tubocurarine, the activity diminished relatively quickly. The tubocurarine had been applied to the right side of the cord for 15 min. Sections *A*, *B* and *C* begin 4, 11 and 14 min,

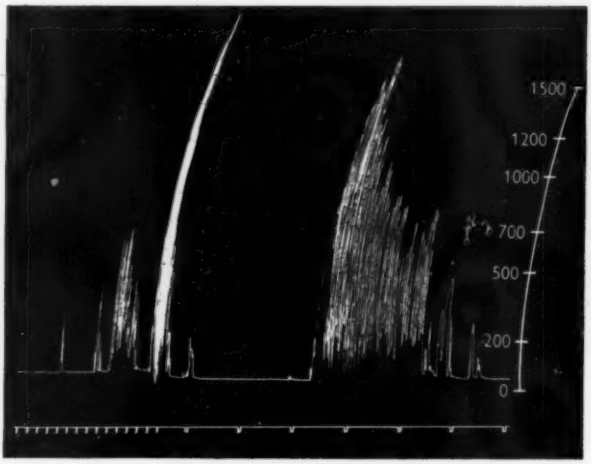

Fig. 5. Record of left anterior tibialis muscle of a cat under pentobarbitone sodium anaesthesia a few minutes after topical application of 0·1 % tubocurarine to the left side of the exposed dorsal surface of the upper cervical cord. A few initial irregular contractions followed by bursts of scratching movements occur, the last one being recorded on a faster moving drum. Frequency of beats: 2/sec. On the right, tension in grams. Time marker, 10 sec.

respectively, after its removal. At 4 min the activity of the right tibialis muscle had not perceptibly diminished; the contractions were as strong as during the time of tubocurarine application. At 11 min the individual beats in each burst have become much weaker and at 14 min there is a further attenuation. At the beginning of section *C* 0·2 % bromophenol blue has been applied to the left side of the spinal cord; 6 min later strong contractions begin in the left tibialis muscle and with the onset of this activity all contractions of the right muscle cease.

In some experiments records from both tibialis muscles were taken after decerebration in pentobarbitone sodium anaesthesia and the tubocurarine was first applied to the left side and later to both sides of the cervical cord. In the experiment of Figs. 7 and 8, decerebration was performed 2 hr

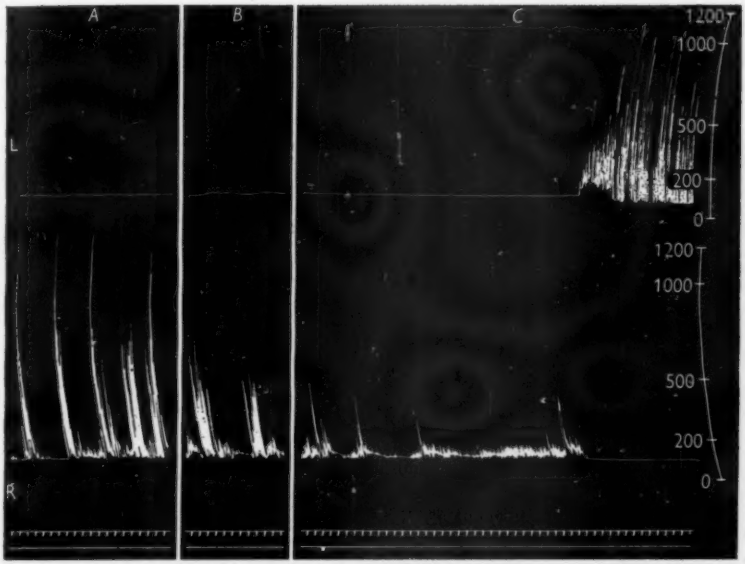

Fig. 6. Records of left (L) and right (R) anterior tibialis muscles of a cat under pentobarbitone sodium anaesthesia. Record *A* begins 4, record *B* 11, and record *C* 14 min after removal of tubocurarine (0·1 %), which had been applied for 15 min to the right side of the exposed dorsal surface of the upper cervical cord. The records show the attenuation of activity in the right muscle which comes to an abrupt end when, on application of 0·2 % bromophenol blue (at the signal on bottom line) to the left side of the exposed dorsal surface of the upper cervical cord, activity begins in the left muscle. On the right, tension in grams. Time marker, 10 sec.

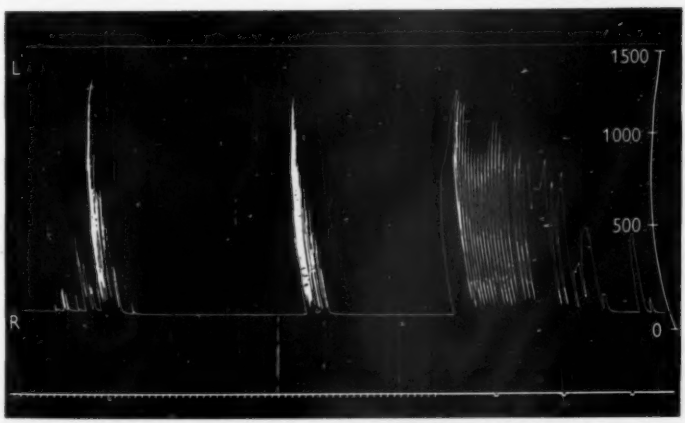

Fig. 7. Records of left (L) and right (R) anterior tibialis muscles of a cat decerebrated under pentobarbitone sodium anaesthesia. Tubocurarine (0·1 %) applied to the right side of the exposed dorsal surface of the upper cervical cord. The record shows the beginning of activity in the right muscle. On the right, tension in grams. Time marker, 10 sec.

after the pentobarbitone sodium injection; Fig. 7 shows the effect of the unilateral application of tubocurarine. After a latency of 14 min, activity begins in the right muscle with a few irregular contractions followed by three discrete bursts of strong rhythmic beats. As the onset of each burst

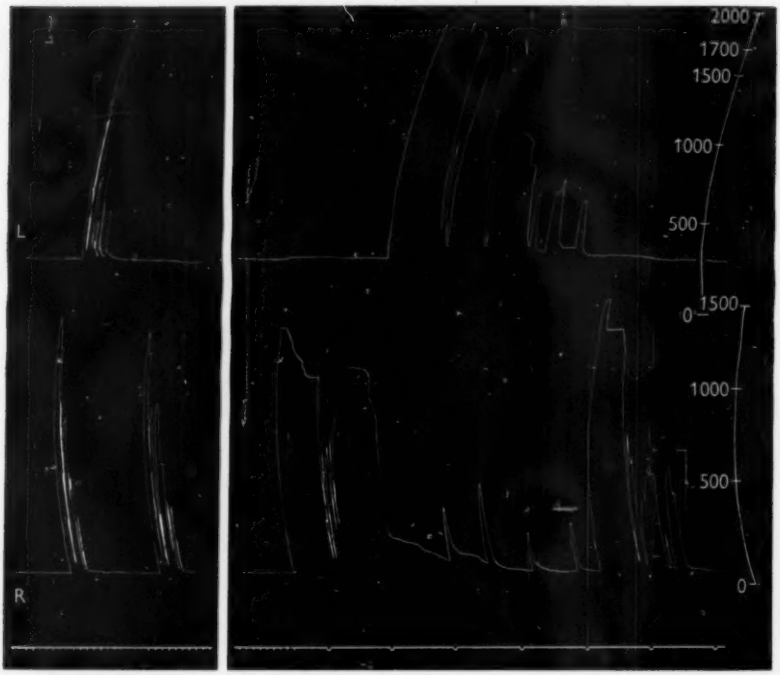

Fig. 8. Records of left (L) and right (R) anterior tibialis muscles of a cat decerebrated whilst under pentobarbitone sodium anaesthesia. Continuation of Fig. 7, 2 hr later. Tubocurarine (0·1 %) applied to both sides of the exposed dorsal surface of the upper cervical cord. On the right, tension in grams. Time marker, 10 sec.

was preceded by a few weak myoclonic movements of the paws it was easy to increase the speed of the drum just before the onset of the third burst and thus to show its individual beats: they occurred at a frequency of about 2/sec. The left tibialis muscle remained inactive throughout the unilateral application of the tubocurarine. When later it was applied to both sides, discrete bursts occurred in both tibialis muscles, but the periods of activity alternated. In this experiment it was found that at this late stage, about 3 hr after the decerebration, the bursts consisted of fewer but longer-sustained contractions. The alternation and this change in the character of the bursts is illustrated in Fig. 8.

In one experiment a transverse section was made with a thermocautery through the medulla oblongata just below the obex. This did not abolish the muscular contractions but they became attenuated and occurred less frequently.

Chloralose anaesthesia

In experiments carried out for another purpose together with A. B. Cairnie and J. L. Malcolm, it was found that the scratching movements, elicited in cats under pentobarbitone anaesthesia by the topical application of bromophenol blue or tubocurarine to the upper cervical cord, did not occur in cats under chloralose anaesthesia, although in this condition tubocurarine still produced muscular effects by acting on more rostral

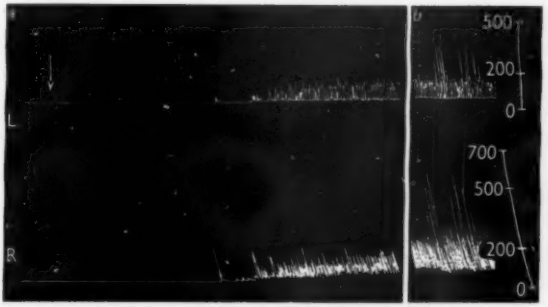

Fig. 9. Records of left (L) and right (R) anterior tibialis muscles of a cat under chloralose anaesthesia. Topical application of 0·1 % tubocurarine to the exposed dorsal surface of the upper cervical cord had produced no muscular activity. At the arrow (↓) 200 μg tubocurarine injected into the cannulated left lateral ventricle. On the right, tension in grams. Time marker, 10 sec.

parts of the neuraxis (Feldberg & Malcolm, 1959). Such an experiment with chloralose anaesthesia is illustrated in Fig. 9. First, tubocurarine 1/1000 was applied to both sides of the exposed dorsal surface of the cervical cord at the region of C1 for 30 min; no contractions of the anterior tibialis muscle or any other muscular action ensued. Then, at the arrow in Fig. 9, 200 μg of tubocurarine was injected through a Collison cannula implanted into the left lateral ventricle. About 5 min later there occurred generalized fasciculation, tremor-like activity and frequent small jerk-like contractions of the shoulders and thorax, which later led to small jerks of the whole animal. There was no indication, however, of either scratching movements or of alternating activity between the right and left hind legs. Figure 9 shows the records taken from both tibialis anterior muscles. At first (at *a*) tremor-like activity only was recorded; later, at *b*, this activity was interspaced by somewhat stronger contractions which reflected the small jerks of the whole animal in these muscles.

The finding that under chloralose anaesthesia no scratching movements are produced by topical application of the tubocurarine to the upper cervical cord cannot be explained by an inability of the tubocurarine to penetrate into the tissue of the spinal cord in this condition, as is evident from the following result. When the scratching movements have been produced under pentobarbitone anaesthesia and the topical application of the drug is continued whilst chloralose 70 mg/kg is injected intravenously, they immediately become attenuated and cease within a few minutes.

DISCUSSION

The finding that in cats under pentobarbitone sodium anaesthesia topical application of either bromophenol blue or tubocurarine to the dorsal surface of the exposed upper cervical cord produces a pattern of muscular activity resembling that seen in the scratch reflex, and that the application to one side of the cord produces the scratching movements in the hind leg on the ipsilateral side, suggests a selective activation of the long descending propriospinal neurones in the lateral column of the spinal cord, which on stimulation of the skin mediate the scratching movements of the hind legs, as was shown by Sherrington & Laslett (1903) in their classical experiments of successive degeneration.

An indirect activation of these neurones by an excitatory action of the substances on the cells of the nuclei gracilis and cuneatus can be excluded, because the scratching movements were also obtained after decerebration whereas the fibres from these nuclei are known to be ascending afferent ones and to relay almost entirely in the contralateral thalamus (Matzke, 1951; Bowsher, 1958). Therefore the substances must act at the synapses where the sensory fibres from the skin impinge either directly or through internuncials on the large descending propriospinal neurones, and the action may well be an excitation or depolarization of the cell bodies of these neurones.

The fact that the topical application of the substances to a restricted area of the cervical cord imitates the whole pattern of movements associated with the scratch reflex, i.e. not only the scratching movements of the hind leg but also the postural changes of the body which are an accompaniment of this reflex and such movements of the foreleg as a cat performs when wiping its face, would be difficult to understand if the substances were to act indiscriminately on all synapses present in this region and reached by the bromophenol blue and tubocurarine when penetrating through the superficial layers of the cord. The action must therefore be a highly selective one confined to those neurones in the upper cervical cord which are activated in the scratch reflex by the afferent impulses from the skin; or, in other words, the action must be selectively on those

neuronal structures which form the 'reflex centre' for the scratch reflex in this region.

Pentobarbitone sodium is generally considered to have a more profound central depressant action than chloralose, which has some of the properties of a convulsant besides those of an anaesthetic (Adrian & Moruzzi, 1939). Yet in chloralose anaesthesia neither bromophenol blue nor tubocurarine elicited the scratching movements. This finding could indicate that chloralose is more depressant on the spinal cord and pentobarbitone on the more rostral parts of the neuraxis. An alternative explanation would be that chloralose has a specific depressant action on the neuronal system that is activated when the topical application of bromophenol blue or tubocurarine elicits the scratching movements. For instance, if no internuncial neurones, or only a few, were taking part in this activation, the results might suggest that the depressant action of pentobarbitone is more on internuncial activity, that of chloralose more on neurones or their cell bodies which form long pathways in the spinal cord, such as the long descending propriospinal fibres.

From the finding that the scratching movements occur in pentobarbitone but not in chloralose anaesthesia, it does not necessarily follow that pentobarbitone has no depressant effect on this phenomenon. It would be sufficient to assume that its depressant action is weaker than that of chloralose. Recent experiments (F. R. Domer & W. Feldberg, unpublished) carried out to investigate this problem have in fact shown that pentobarbitone, too, exerts a depressant effect on the scratching movements obtained by the topical application of tubocurarine. These experiments made it also possible to explain why, in one of the present experiments, the bursts of activity obtained with tubocurarine in a cat decerebrated in pentobarbitone sodium anaesthesia consisted of more sustained muscular contractions. It was found that pentobarbitone could convert such sustained contractions into typical bursts of fast rhythmic activity. It is therefore reasonable to conclude that in the specific experiment described in this paper the effect of pentobarbitone had worn off, particularly since the sustained contractions were obtained 3 hr after the decerebration, which again was performed 2 hr after the pentobarbitone sodium injection. This experiment thus revealed the type of muscular activity which may occur on the topical application of tubocurarine in the unanaesthetized decerebrate cat.

Although both bromophenol blue and tubocurarine elicit the scratching movements when applied to the upper cervical cord, they differ pharmacologically in their peripheral as well as in their central effects. Bromophenol blue lacks not only the neuromuscular blocking action (K. Fleischhauer, unpublished experiments), but also the other central motor effects which

tubocurarine exerts on more rostral parts of the neuraxis. In fact, as was stated in the introduction, the starting point for the present experiments was the observation that bromophenol blue, unlike tubocurarine, did not produce muscular contractions when perfused through the cerebral ventricles, but did so when it reached the subarachnoid space. These observations were made in cats anaesthetized with pentobarbitone sodium. It is naturally not certain whether the same condition pertains in unanaesthetized cats. In these the injection of bromophenol blue into the cerebral ventricle produces strong muscular effects of a convulsive type (unpublished experiments). It would be interesting to know whether this effect is entirely the result of the dye reaching the subarachnoid space, or if there is in addition some contribution due to an action on the grey matter surrounding the aqueduct and the cerebral ventricles. If the effect were entirely one produced on the spinal cord when the dye reaches the subarachnoid space, it would mean that, at least in the cat, a convulsive type of activity can result from an action of drugs activating descending pathways in the upper cervical cord, and that exaggerated scratching movements may form the basis of a convulsive activity. These considerations apply also to a number of other drugs which on injection into the cisterna magna produce convulsions.

Finally, it must be pointed out that the excitatory action of tubocurarine on the upper cervical cord which results in the scratching movements is not the cause of the striking changes in electrical activity of the brain which occur when this substance is injected into the cerebral ventricles and which resemble so closely the seizure discharges of epilepsy. This is evident from the fact that the electrical changes occur also in cats that are anaesthetized with chloralose (Feldberg, Malcolm & Smith, 1957), although in this condition tubocurarine does not elicit the scratching movements. Further A. B. Cairnie and J. L. Malcolm (personal communication) obtained the electrical changes also when tubocurarine was perfused from the lateral cerebral ventricle to the aqueduct and did not reach the subarachnoid space. On the other hand, the electrical changes could not be reproduced by the topical application of tubocurarine to the upper cervical cord, as was shown in preliminary experiments together with Cairnie and Malcolm.

SUMMARY

1. There is a restricted region of the upper cervical spinal cord, at the level of C 1, from which it is possible to elicit regularly, in cats anaesthetized with pentobarbitone sodium, scratching movements of the hind limbs by topical application to the dorsal surface of either 0·2 % bromophenol blue or of 0·1 % tubocurarine. The scratching movements are associated with

postural changes, the attitude being that of a cat scratching itself behind the ear. Further, the forelegs perform movements which resemble those which a cat performs when wiping its face.

2. The scratching movements of the hind legs were recorded myographically from the anterior tibialis muscles.

3. The scratching movements occurred usually in bursts. When the topical application of bromophenol blue or of tubocurarine was to one side of the cord they occurred, with the associated movements of the foreleg, on the ipsilateral side only. When the topical application was to both sides, bursts occurred alternately on either side.

4. Decerebration at mid-collicular level did not abolish the motor effects obtained on topical application of either bromophenol blue or tubocurarine to the dorsal surface of the upper cervical cord.

5. In chloralose anaesthesia motor effects could not be elicited on topical application of either bromophenol blue or tubocurarine to the dorsal surface of the upper cervical cord.

REFERENCES

Adrian, E. D. & Moruzzi, G. (1939). Impulses in the pyramidal tract. *J. Physiol.* **97**, 153–199.

Bowsher, D. (1958). Projection of the gracile and cuneate nuclei in Macaca mulatta: an experimental degeneration study. *J. comp. Neurol.* **110**, 135–151.

Feldberg, W. & Fleischhauer, K. (1960). Penetration of bromophenol blue from the perfused cerebral ventricles into the brain tissue. *J. Physiol.* **150**, 451–462.

Feldberg, W. & Malcolm, J. L. (1959). Experiments on the site of action of tubocurarine when applied via the cerebral ventricles. *J. Physiol.* **149**, 58–77.

Feldberg, W., Malcolm, J. L. & Smith, I. D. (1957). Effect of tubocurarine on the electrical activity of the cat's brain under chloralose. *J. Physiol.* **138**, 178–201.

Matzke, H. A. (1951). The course of the fibres arising from the nucleus gracilis and cuneatus of the cat. *J. comp. Neurol.* **94**, 439–452.

Merlis, J. K. (1940). The effect of changes in the calcium content of the cerebrospinal fluid on spinal reflex activity in the dog. *Amer. J. Physiol.* **131**, 67–72.

Sherrington, C. S. & Laslett, E. E. (1903). Observations on some spinal reflexes and the interconnection of spinal segments. *J. Physiol.* **29**, 58–96.

Winkler, C. & Potter, Ada (1914). *An Anatomical Guide to Experimental Researches on the Cat's Brain.* Amsterdam: W. Versluys.

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EFFECTS OF CHANGES IN EXTRACELLULAR CALCIUM CONCENTRATION ON THE POTASSIUM-INDUCED CONTRACTURE OF FROG'S SKELETAL MUSCLE

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The exact nature of the process or processes which link the electrical and mechanical events in muscle activity remain unknown. Evidence indicating that calcium ions are in some manner involved in this process has been summarized by Sandow (1952). As a working hypothesis, he suggested that an action potential or depolarization of the muscle fibre surface permits or promotes the entrance of calcium ions into the fibre and that these ions then initiate the mechanical events (Sandow, 1952). The demonstration by Hodgkin & Keynes (1957) that an influx of calcium ions accompanies each action potential in the squid giant axon encouraged investigation of this hypothesis. Recently, a similar influx of calcium into frog's skeletal muscle during activity has been demonstrated by Bianchi & Shanes (1959).

In a preliminary note (Frank, 1958), it has been reported that soaking the extensor longus digiti IV muscle of the frog for a few minutes in a calcium-free solution will prevent the development of a potassium-induced contracture in this muscle. Treatment of the muscle with the calcium-free solution did not reduce the depolarization produced by an increased potassium concentration, normally effective in inducing the contracture. Evidence is now presented that the speed with which the potassium-induced contracture is eliminated is determined by the rate at which calcium ions leave the extracellular spaces of the muscle.

METHODS

Preparation. The extensor longus digiti IV muscle of the frog, *Rana pipiens*, was used in most experiments. This muscle was removed with pieces of adjacent muscles attached and dissected free under a dissecting microscope. The loosely fitting sheath surrounding the muscle was also removed. The tendons at each end of the muscle are attached to the sheaths of other muscles. These attachments and adjacent connective tissues were left intact, to permit tying with minimum damage to the muscle. After isolation this muscle has the shape of a cylinder approximately 20 mm long with either a circular cross-section (diameter,

0·26–0·42 mm) or an elliptical cross-section (major axis, 0·27–0·70 mm and minor axis 0·19–0·49 mm). In a few experiments the extensor longus digiti IV muscle of the bullfrog, *Rana catesbeiana*, was used. Unless otherwise noted all results refer to experiments performed on muscles from *Rana pipiens*.

Solutions. The primary solution had the following composition (mM): choline chloride 111·8; KCl 2·47; $CaCl_2$ 1·08; $NaHCO_3$ 2·38; NaH_2PO_4 0·087; glucose 11·1. Except for the absence of the $CaCl_2$, the composition of the calcium-free solution was identical. Solutions with intermediate calcium concentrations were prepared by mixing appropriate amounts of the above two solutions. Solutions with elevated calcium or potassium concentrations were prepared by the addition of solid $CaCl_2$ or KCl to the primary solution. Exceptions were the isotonic potassium chloride solutions, which contained 123 mM-KCl with or without 1·08 mM-$CaCl_2$. In some experiments the solutions contained NaCl in the place of choline chloride. The cases in which sodium solutions were used will be specifically pointed out in the Results. The caffeine (Kahlbaum) was dissolved in a small quantity of solution with the aid of a few drops of concentrated HCl and diluted to the desired concentration. The resulting caffeine solution was buffered to pH 7·2 by the addition of solid $NaHCO_3$. In all solutions D-tubocurarine chloride (Burroughs Wellcome) was added to make a final concentration of 10^{-4} g/ml. The solutions were stored in polyethylene bottles in order to minimize contamination.

Water for the above solutions was prepared by passing distilled water through a Bantam Demineralizer (Barnstead Still and Sterilizer Co.). The conductivity of this purified water was less than that of a 0·0017 mM-NaCl solution. All glass-ware was rinsed thoroughly with this purified water and care was exercised during the experiments to prevent contamination with calcium from any source.

Mechanical recording. The muscle was mounted vertically in a cylindrical bath of 10 ml. capacity. Solutions were introduced into the bath at the top with a syringe and removed via an outlet at the bottom. The lower end of the muscle was fixed and the upper end was attached by means of a nylon thread to the free end of a lever approximately 3 in. (7·5 cm) above the bath. Two strain gauges (Baldwin–Lima–Hamilton Corpn, SR_4 Type A-7) were mounted on both sides of a brass plate near the fixed end of the lever arm. These formed two series arms of a Wheatstone bridge. The output of the latter was fed into a Grass Model 5P1, low-level DC pre-amplifier and recorded with a Grass Model 5 ink-writing oscillograph. A 0·5 g weight displaced the lever arm 0·25 mm. This was equal to a muscle shortening of about 1·25% and, and since the muscles rarely develop so great a tension, the recordings were considered to be essentially isometric. The resting tension was about 0·5 g in all experiments.

Electrical recording. The muscle was mounted horizontally in a rectangular bath. A bipolar stimulating electrode, a ground electrode and a bipolar recording electrode, all constructed of platinum wire, were placed along the muscle. Before recording, the solution was removed from the bath by suction. Supramaximal rectangular stimulating pulses 1 msec in duration were used. The compound action potential was recorded with DC amplification, displayed on a cathode-ray oscilloscope and photographed. The amplitude of the initial negative phase of the diphasic action potential was taken as a measure of the number of the muscle fibres responding to the stimulus. In one experiment the muscle under one pole of the recording electrode was crushed and a monophasic action potential was recorded.

Procedure. In all but a few of the experiments tests consisted of determining the effects of altering the ionic environment of the muscle on the potassium-induced contracture. After isolation the muscle was kept in the primary solution (see above) for at least 30 min before testing. The contracture (Fig. 1) was produced by replacing this with a solution containing 25 mM potassium. Near the end of the mechanical response the test solution was replaced by the primary solution in which the muscle was kept for at least 10–15 min before another test was performed. After an initial equilibrium period of about 1 hr reasonably

reproducible contractures were obtained. However, in a few muscles the response gradually increased for several hours.

To determine the effects of altering the ionic environment on the contracture, the muscle was soaked in the altered solution for a predetermined time (pre-test soaking period) and then tested with the altered solution containing 25 mM potassium. In order to eliminate, if possible, all sources of calcium contamination, when soaking the muscle in a low calcium or a calcium-free solution the bath was emptied approximately 10 sec after the initial exposure to the altered solution and fresh altered solution was placed in the bath. Another replacement of the altered solution was carried out approximately half way through the pre-test soaking period. Between each test with altered solutions, a control contracture of the muscle was obtained using the primary solution containing 25 mM potassium. The response immediately preceding a test in an altered solution was used as control. Whenever a control response reasonably similar to previous controls was not obtained the experiment was abandoned. However, as judged from the control contractures, this preparation almost always survived in good condition for 5–6 hr of testing.

THEORETICAL

When a cylinder containing an initial concentration of a substance, C_0, is placed in an infinitely large volume of solution having a zero concentration of the substance, the concentration, C', remaining in the cylinder at time t, will depend upon the diffusion coefficient, D, of the substance, the dimension of the cylinder and conditions at the surface of the cylinder (Crank, 1956). Two limiting conditions occur. One when the surface concentration is kept at 0 and the other when the cylinder and the surrounding solution is undisturbed. The actual conditions of the present experiments lay somewhere between these two extremes, because it was necessary to disturb the system in order to reduce calcium contamination and to produce a contracture and vigorous stirring of the external solution would have made it impossible to record small responses.

The actual conditions of the experiments came closer to the undisturbed system. A solution for the diffusion equation for this condition and for large values of t is

$$\frac{C'}{C_0} = \frac{R^2}{4Dt}, \tag{1}$$

where R is the radius of the cylinder. This relation is plotted in Fig. 3. Also under either condition

$$\frac{C'}{C_0} = f(R^2/Dt). \tag{2}$$

Using this relation it can be shown that

$$\frac{\log t_2 - \log t_1}{\log (c_2/A_2) - \log (c_1/A_1)} = -2, \tag{3}$$

where the subscripts 1 and 2 refer to different muscles; t_1 and t_2 are the diffusion times at $C'_1/C_{01} = C'_2/C_{02}$. c is the circumference of the muscle;

and A is the cross-sectional area. Since the toe muscles used in this work often had elliptical cross-sections, the value c/A was used rather than the value R. These are related by $c/A = 2/R$.

Thus, provided the percentage inhibition of maximum contracture tension is related to the change in calcium concentration in various muscles, the time that toe muscles need to be kept in a calcium-free solution in order to produce a certain percentage inhibition would be linearly related to their c/A's when these values are plotted on log-log. scales (Fig. 5).

RESULTS

Effects of calcium-free solutions. In a previous communication (Frank, 1958) it was reported that soaking the toe muscle of the frog in a calcium-free solution consistently inhibited the potassium-induced contracture of the muscle. A typical experiment is shown in Fig. 1. The rectangular artifact at the start and end of each response was produced by emptying the bath. It took approximately 0·5 sec to fill the bath with the new solution and thus the filling was completed during the downstroke at the end of the rectangular artifact. Each test involving the use of calcium-free solutions was preceded and followed by a control response, the only exception being the final test response in a series. Although in the experiment shown in Fig. 1 complete elimination of the response was not followed by a control response, this was not the usual case. In all the muscles so tested a normal control response was obtained when the muscle was kept in the primary solution for a sufficient length of time following a test response. It should also be pointed out that between the responses in Fig. 1 *F* and *G* another experiment was carried out using this same muscle.

Although in all the experiments presented below the contractures were induced by a solution containing 25 mM potassium, contractures induced by an isotonic (123 mM) potassium chloride solution also were eliminated by first treating the muscle with a calcium-free solution. An experiment in which an isotonic potassium chloride solution was used is shown in Fig. 2. Eight experiments with isotonic potassium chloride have been performed and as far as could be determined the time course for the inhibition produced by calcium-free solutions did not differ from experiments in which the contracture was induced by 25 mM potassium.

It should be pointed out that in the experiment illustrated (Fig. 2) the maximum tension of the control responses averaged about 1 g. The cross-sectional area of this muscle was 0·041 mm² and thus the maximum tension of the contracture induced by isotonic potassium chloride averaged 2·44 kg/cm². This is quite close to the maximum tetanic tension of the frog's sartorius muscle, reported by Hajdu (1951) to be 2·5 kg/cm². Thus there can be little doubt that all the muscle fibres were maximally activated during the contracture and that the response of all fibres was eliminated by treating the muscle with a calcium-free solution.

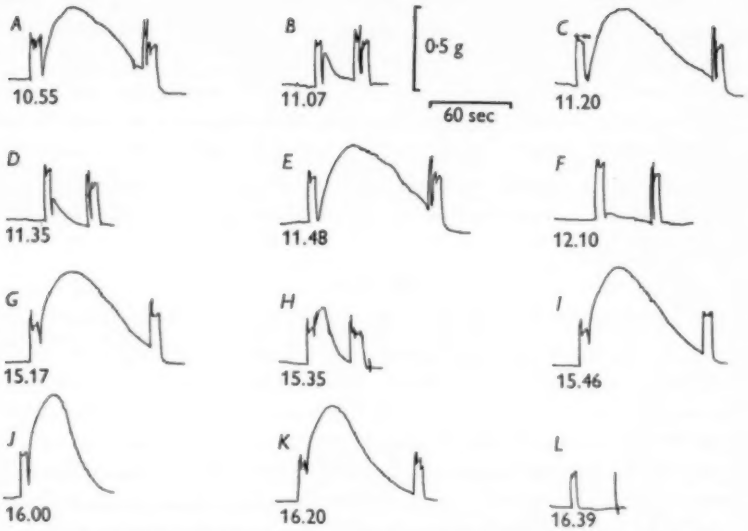

Fig. 1. Inhibition of contractures produced by 25 mM potassium in the frog's toe muscle by soaking the muscle in a calcium-free solution. *A*, *C*, *E*, *G*, *I*, and *K* control responses. Time (min) that muscle kept in a calcium-free solution before testing with a calcium-free solution containing 25 mM potassium *B*, 2; *D*, 5; *F*, 10; *H*, 1; *J*, 0; and *L*, 15. Rectangular artifacts at start and end of each response caused by emptying the bath. Bath filled during the downstroke at the end of the artifact. Time of day recorded below each test record. Muscle cross-section 0·10 mm².

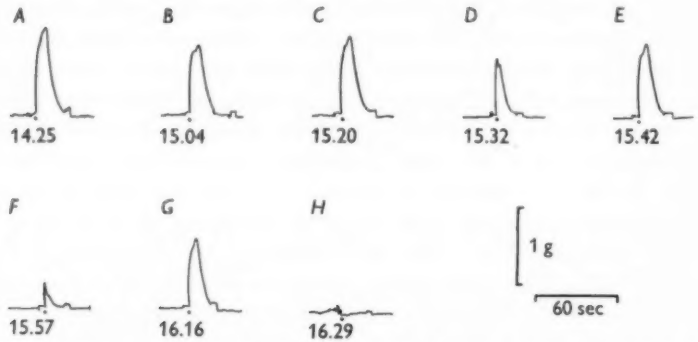

Fig. 2. Inhibition of contractures produced by isotonic potassium chloride in the frog's toe muscle by soaking the muscle in a calcium-free solution. *A*, *C*, *E*, and *G* control responses produced by a solution containing only 123 mM potassium chloride and 1·08 mM calcium chloride. Time (min) that muscle was kept in a calcium-free solution before testing with a 123 mM potassium chloride solution, *B*, 0; *D*, 1; *F*, 3; and *H*, 6. Dots immediately below each record indicate point at which potassium chloride solutions put in bath. Time of day recorded below each test record. Muscle cross-section 0·041 mm².

In all of five small toe muscles (cross-sectional areas less than 0·10 mm²) tested the maximum tension of the contracture induced by isotonic potassium chloride was at least as large as the maximum tetanic tension of the same muscle.

In order to eliminate the complicating effect on excitability of altering the calcium concentration, all experiments with potassium-induced contractures were performed with solutions which contained choline rather than sodium. However, the potassium-induced contracture also was eliminated by soaking the muscle in a calcium-free solution when the solutions contained sodium in place of choline. In the latter case the complete elimination of the response took about one third longer than when choline solutions were employed. When using sodium solutions it was essential that they should contain adequate concentrations of tubocurarine. This may account for the differences in the results reported here and those previously reported by Denton (1948). The small residual response in the absence of curare is dependent upon the cations present in the bathing solution. It is being investigated and results will be reported later.

In different frog toe muscles, a 50% inhibition of the contracture produced by 25 mM potassium required from 0·5 to 7·8 min of pre-test soaking in the calcium-free solution. The marked differences in the time course of the inhibition in various muscles could be eliminated if, as is shown in Fig. 3, the percentage inhibition is plotted against the time (t) divided by the pre-test soaking period needed for 50% inhibition of the response ($t_{\frac{1}{2}}$). Also included in Fig. 3 is the theoretical curve for the diffusion of a substance from an infinitely long cylinder into an unstirred surrounding medium of infinite dimensions having initially an 0 concentration of the substance (see Theoretical). This curve was fitted to the data on the assumption that the contracture was 50% inhibited when 94% of the calcium, which would have left the muscle at the time the response was 100% inhibited, had diffused out of the muscle (Fig. 8). Despite the differences in the conditions on which the theoretical curve is based and in the experimental conditions, the experimental points fit the theoretical curve reasonably well. This result would seem to suggest that the time course of the inhibition is determined by the rate at which calcium ions diffuse out of the muscle.

Further support for the idea that the time course of the inhibition is determined by a simple diffusion process is provided by the similarity of the time courses of the inhibition and of the recovery from inhibition. This similarity is shown in Fig. 4. In the experiment illustrated, it was first found that the maximum tension was 96% inhibited if the muscle was kept for 1 min in the calcium-free solution before testing. Recovery was then determined in subsequent tests by first soaking the muscle for 1 min in the calcium-free solution and then placing it in the primary

solution for various times before inducing a contracture with potassium. The difference between the inhibition observed and 96% inhibition is plotted as percentage recovery.

As mentioned above, the speed with which the inhibition developed varied greatly in muscles from different frogs. If the time course for the inhibition is determined by the rate at which calcium ions leave the extracellular space when the muscle is placed in a calcium-free solution, the time course would be expected to be related to the dimensions of the muscle. Thus the larger the diffusion area (or the circumference of the

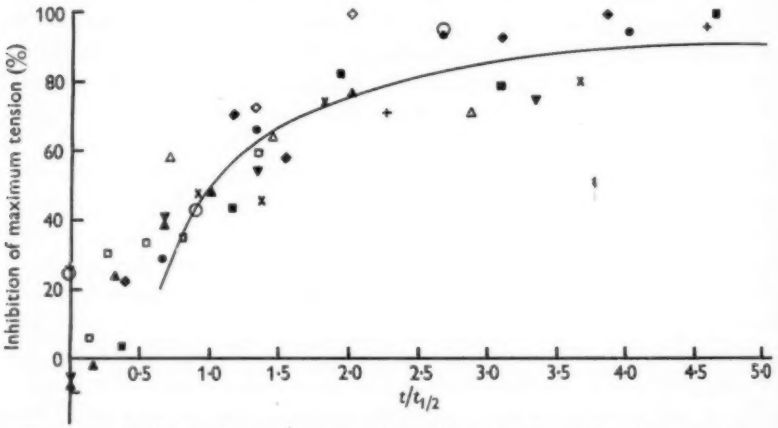

Fig. 3. Reduction in the maximum tension of the potassium-induced contracture of the frog's toe muscle produced by soaking the muscle in a calcium-free solution. Experiments with 11 muscles represented by different symbols. t, time muscle kept in the calcium-free solution before testing with 25 mM potassium; $t_{\frac{1}{2}}$, time for a 50% inhibition of maximum tension (range, 0·5–7·8 min). The line is the theoretical diffusion curve for the change in the concentration of a substance in a cylinder bathed in an infinitely large volume of a solution having a zero concentration of the substance. See text for further details.

muscle, c) the faster the calcium would leave the muscle and therefore the faster the inhibition would develop; whereas the larger the volume of the muscle (or the cross-sectional area, A) the slower the inhibition would develop. It can be shown (Theoretical) that when the time course of the inhibition is related to the dimensions of the muscle, the time for 50% inhibition ($t_{\frac{1}{2}}$) is linearly related to the circumference of the muscle divided by its cross-sectional area (c/A) with a slope of -2 when these values are plotted on a log-log. scale. Since it proved difficult to obtain *Rana pipiens* toe muscles having a low $c:A$ ratio, it was necessary to use the toe muscle of the bullfrog (Fig. 5, open circles) to cover an adequate range for analysis. The results with both species are shown in Fig. 5, where the solid line is

the expected line (slope = −2) and the interrupted line is the regression line of log $t_{\frac{1}{2}}$ on log c/A. The slope of the experimental line is significantly different from 0 ($P < 0{\cdot}01$) and is not significantly different from the theoretical line ($P > 0{\cdot}5$; student's t test).

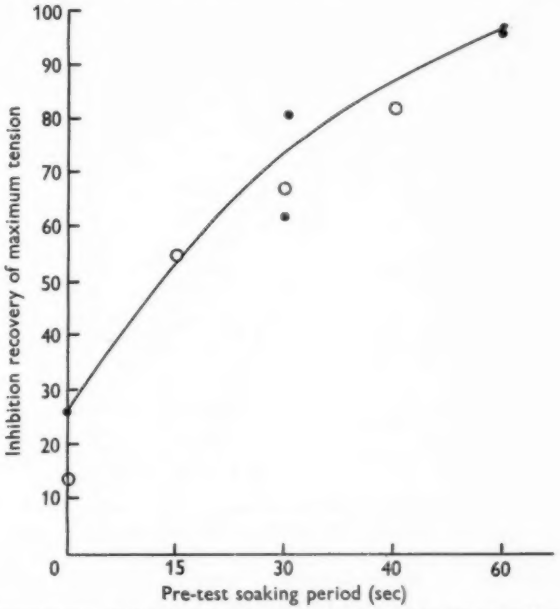

Fig. 4. Comparison of the development of the inhibition of potassium-induced contractures produced by placing a toe muscle in a calcium-free solution (●) with the recovery produced by placing the muscle in a solution having 1·08 mM calcium following 1 min in a calcium-free solution (○).

Onset of the inhibition. When the toe muscle is placed in the calcium-free solution, one would expect the fluid surrounding the fibres at the surface of the muscle to be rapidly depleted of calcium ions, while the calcium concentration of the extracellular fluid in the centre of the muscle would decrease more slowly. It was therefore of interest to determine how soon an inhibition of tension could be seen after the muscle was placed in the calcium-free solution, in order to get some estimate of the speed with which the inhibition develops in the individual muscle fibres. In four of the experiments the maximum tension was inhibited by 10–26 % when the contracture was produced by a high-potassium, calcium-free solution without previously soaking the muscle in the calcium-free solution. These inhibitions developed during the 10–15 sec necessary for the contracture to reach its maximum (Fig. 6*A*).

However, more commonly, an increase of the maximum tension of the contracture was observed when the muscle was tested after a short exposure (less than 1 min) to the calcium-free solution (Fig. 6*B*). This increase was always accompanied by a decrease in the duration of the

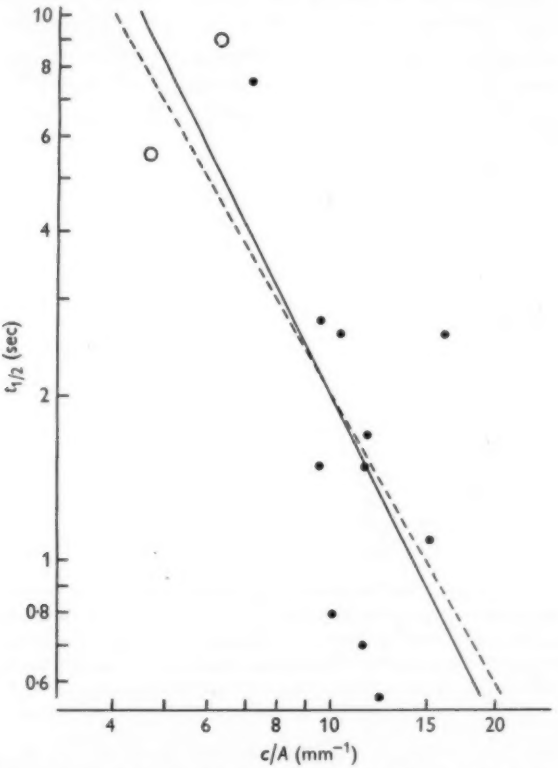

Fig. 5. Relation between the cross-sectional dimensions of toe muscles and the time ($t_{\frac{1}{2}}$) that they had to be kept in a calcium-free solution in order to produce 50% inhibition of the maximum tension of the potassium-induced contracture. Muscles from *Rana pipiens* (●); muscles from *Rana catesbeiana* (○); solid line, theoretically expected on basis of diffusion relations (see Theoretical); interrupted line, regression line of log $t_{\frac{1}{2}}$ on log c/A; circumference/cross-sectional area, (c/A); log-log. scale.

rising and falling phases of the contracture. With longer pre-test soaking periods in the calcium-free solution the contracture tension was always either reduced or completely suppressed.

Effects of changes in the external calcium concentration on the potassium-induced contracture. The observations just described clearly show that as the calcium ion concentration in the fluid surrounding the muscle fibres is

reduced the fibres pass through a phase in which their mechanical response is potentiated. In order to appreciate the changes which occur when the toe muscle is soaked in a calcium-free solution, it is necessary to know the changes in tension developed by the muscle fibres when the calcium ion concentration in the fluid surrounding the individual fibres is altered.

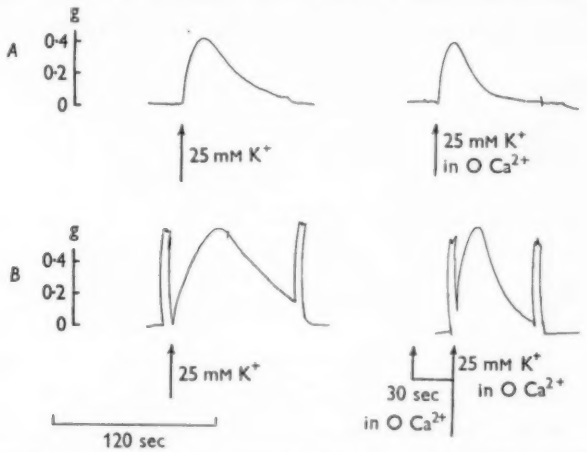

Fig. 6. Typical changes in the potassium-induced contracture of toe muscles produced by short exposures to calcium-free solutions. Control responses on the left. *A* and *B* different muscles. Square-wave-like traces at the start and end of the contractures in *B* are artifacts produced by changing the solution in the bath.

In the experiment presented in Fig. 7, it was found that keeping the muscle for 1·5 min in a solution containing only 0·054 mM calcium inhibited the contracture by about 55 %. It took the same length of time to eliminate the mechanical response completely when this muscle was treated with the calcium-free solution. Soaking the muscle for more than three times this length of time in the 0·054 mM calcium solution did not produce a significant further reduction in maximum tension. Thus it would seem that an equilibrium was achieved between the Ca^{2+} ion concentrations of the bathing solution and some location in the muscle resulting in a change in the tension of the contracture.

The above observation made it possible to study the mechanical response of the muscle after equilibration with a solution of altered calcium concentration had been achieved. The time that the toe muscle had to be kept in the calcium-free solution in order to obtain 100 % inhibition was first determined. Next, the muscle was placed in a solution having an altered calcium concentration for the same length of time and then the potassium-induced contracture was tested. The results from experiments

of this type were variable (Fig. 8). However, as is shown by the line drawn through the means, a consistent pattern appeared. As the calcium concentration of the bathing solution was reduced there was a phase in which the maximum tension of the contracture was increased. From 0·108 to 0 mM calcium, the maximum tension decreased in a regular fashion and even at 0·01 mM calcium a mechanical response was still present. The durations of all contractures induced with the muscle bathing in a solution

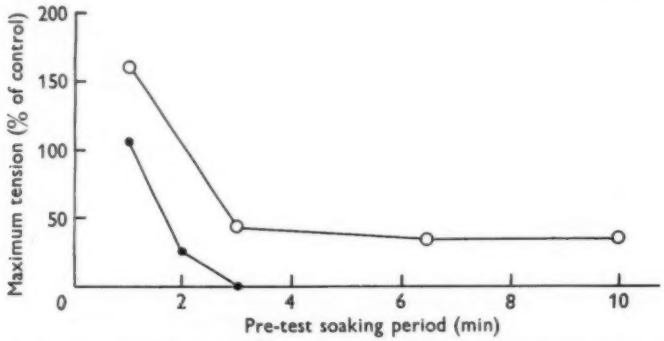

Fig. 7. Changes in the maximum tension of the potassium-induced contracture of a toe muscle produced by exposing the muscle to either a calcium-free solution (●), or to a solution containing 0·054 mM calcium (○).

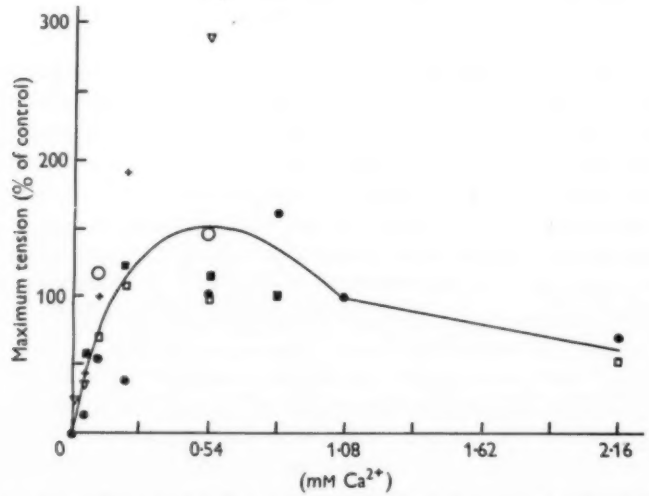

Fig. 8. The maximum tension of potassium-induced contractures after equilibrium with various calcium concentration in the bathing solution. Six toe muscles represented by different symbols. Line drawn through the means.

having less than the usual calcium concentration (1·08 mM-Ca^{2+}), were reduced.

When the calcium concentration was doubled (2·16 mM-Ca^{2+}) there was a small reduction in the maximum tension of the contracture. However, the most striking change produced by this treatment was a large increase in the duration of the response. Contractures lasting more than three times as long as the controls were obtained in this manner.

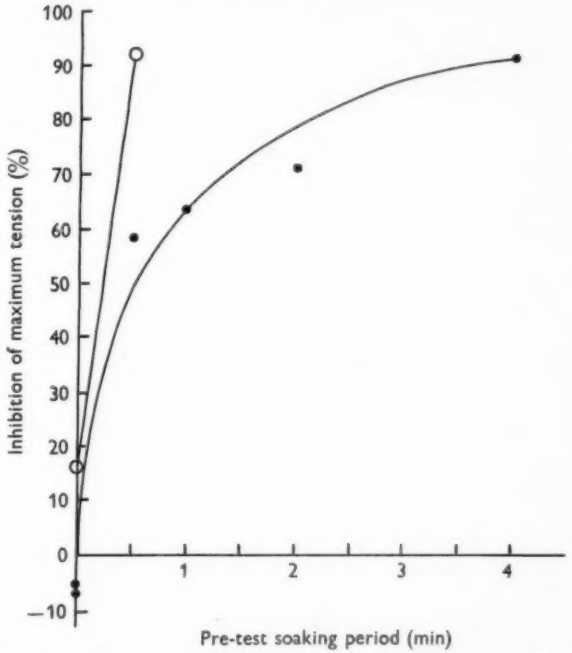

Fig. 9. Effect of ethylenediamine tetraacetic acid (EDTA) on the inhibition of the potassium-induced contracture produced by treating a toe muscle with a calcium-free solution. (●), calcium-free solution; (○), calcium-free solution containing 1·08 mM-EDTA.

Effect of EDTA on the inhibition. The disodium salt of ethylenediamine tetraacetic acid (EDTA) is a chelating agent which rapidly and firmly binds calcium ions (Chenoweth, 1956) and there is evidence that its distribution is limited to the extracellular space (Leckie & Tompsett, 1958). It was found that the addition of a small amount of EDTA to the calcium-free solution greatly accelerated the rate with which the inhibition could be produced (Fig. 9). In the experiment illustrated, 92% inhibition was obtained eight times faster when 1·08 mM-EDTA was added to the calcium-free solution.

Inhibition of the action potential. Recently it has been demonstrated that placing nerves (Frankenhaeuser & Hodgkin, 1957; Frankenhaeuser, 1957) or skeletal muscles (Ishiko & Sato, 1957; Edwards, Ritchie & Wilkie, 1956) in a calcium-free solution quickly renders these tissues inexcitable. Although information concerning this phenomenon is meagre, there is little doubt that it is the result of a loss of calcium ions from a location at or very near the surface of the cells. For this reason it was of interest to compare the time course of the development of inexcitability (of the muscle) with the time course of inhibition of the contracture.

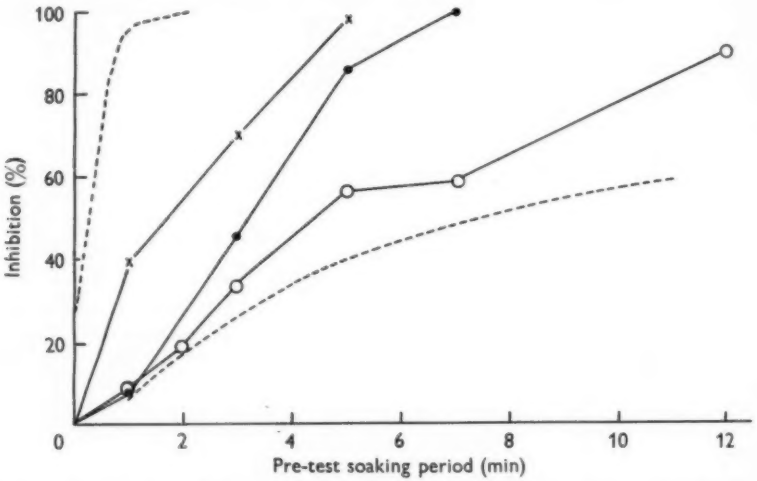

Fig. 10. Comparison of the rate of inhibition of the compound action potential of toe muscles (solid lines), with that for inhibition of the maximum tension of the potassium-induced contracture (interrupted lines) both produced by keeping the muscles in a calcium-free solution for various periods of time. The compound action potential was studied in three toe muscles represented by different symbols. The interrupted lines represent the extremes observed during the course of this investigation. Each symbol represents a separate test preceded and followed by a control test in the primary solution.

Such a comparison is given in Fig. 10. The procedure adopted for this experiment was the same as that described above, with the exceptions that sodium replaced choline in all the solutions, supramaximal electrical stimuli replaced the 25 mM potassium, and the electrical rather than the mechanical response was recorded. Thus control responses were obtained between successive tests in the calcium-free solution. In two of the three muscles the controls, following the tests which resulted in 100 % inhibition, were reduced in amplitude; all other control responses were unmodified. The time courses for the two phenomena (Fig. 10) are remarkably alike.

This would indicate that both time courses are determined by the rate at which calcium ions are lost from the same location in the muscle.

The caffeine contracture. Axelsson & Thesleff (1958) have shown that caffeine contractures can be produced in a skeletal muscle depolarized with potassium. This would indicate that caffeine activates the contractile mechanism of the muscle cell by acting on some process which normally occurs after depolarization or by initiating some process which normally does not occur during contraction. It also suggests the possibility that caffeine contractures can be produced in a calcium-free solution at a time

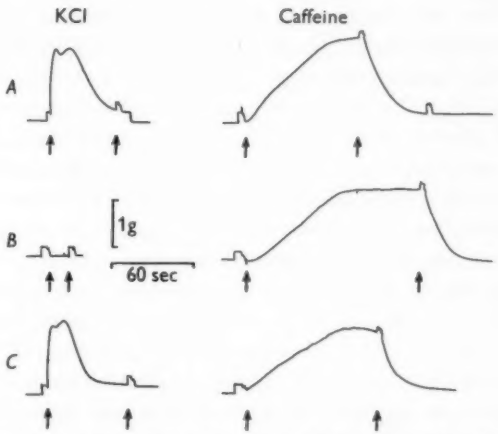

Fig. 11. Comparison of the effects of soaking a frog's toe muscle in a calcium-free solution on the potassium-induced and on the caffeine-induced contractures. *A*, control responses. *B*, tests after muscle kept in a calcium-free solution for 28 min, KCl; and for 31 min, caffeine. *C*, control responses with muscle in primary solution for 40 min after *B*. KCl = 123 mM potassium chloride. Caffeine solution containing caffeine $0{\cdot}5 \times 10^{-4}$ g/ml. Rectangular artifacts caused by emptying the bath. Test solutions in bath between arrows.

when the potasssium contracture is completely inhibited. The effects of soaking a toe muscle in a calcium-free solution on the two types of contractures are shown in Fig. 11. In Fig. 11 *B* it can be seen that an essentially unmodified caffeine contracture can be produced in a calcium-free solution, whereas the potassium-induced contracture is completely suppressed. This result, which was obtained in each of four experiments, shows that the response of the contractile mechanism to an appropriate stimulus can be unchanged although the potassium-induced contracture is completely eliminated by bathing the muscle in a calcium-free solution.

DISCUSSION

The idea that calcium ions play an essential part in muscular contraction is not new. In cardiac and skeletal muscles an action potential normally precedes and initiates the mechanical response. It is possible, and indeed probable, that calcium ions play an essential part in several of the steps leading to contraction. For simplicity we can divide this process into three stages: the action potential, the mechanical response, and the link between the action potential and the mechanical response. We are here concerned with the role of calcium ions as an essential part of the link between electrical and mechanical events in contraction.

In 1883 Ringer demonstrated that the frog heart fails to contract when calcium ions are absent from its perfusion fluid. Later it was shown that under this condition the rhythmic spontaneous action potentials of this tissue are still present in an only slightly modified form (Mines, 1913). The latter observation has been confirmed by using intracellular microelectrode recording techniques (Ware, Bennett & McIntyre, 1955).

One obvious explanation of these observations is that the action potential permits or promotes the movement of calcium ions from the surface to the interior of the cardiac fibres and that these ions then initiate the contraction. This explanation is strongly supported by the work of Niedergerke (1956*a*, *b*, 1957; Niedergerke & Lüttgau, 1957; Lüttgau & Niedergerke, 1958) who studied the effects of altering the ionic environment of cardiac muscle on its electrical activity and mechanical responses.

The striking effects of altering the external calcium concentration on the electrical activity of skeletal muscles have tended to obscure the role of calcium ions as a link between electrical and mechanical events in contraction. Thus, although the tension developed by a skeletal muscle in response to an action potential is markedly reduced when the muscle is bathed in a solution having 5% of the usual calcium concentration, this reduction has been ascribed to a form of fatigue caused by the spontaneous repetitive action potentials which occur under the same conditions (Bülbring, Holman & Lüllmann, 1956). Similarly, the suppression of action potential formation in a calcium-free solution is sufficient to account for loss of the mechanical response to electrical stimulation which simultaneously occurs (Edwards *et al.* 1956). It is for these reasons that in the present study the potassium-induced contracture has been used as a model for the events which occur during a normal contraction. It was assumed that the events leading to the mechanical response of a skeletal muscle during an action potential or a depolarization type of contracture are the same. This concept was probably first suggested by Biedermann (1896) and a considerable body of evidence supporting it has

since accumulated (Gasser, 1930; Kuffler, 1946; Sandow, 1947, 1952; Sten-Knudsen, 1954).

In the present study an attempt has been made to break the link between electrical and mechanical events in contraction by soaking a skeletal muscle in a calcium-free solution. Under the specified conditions it has been shown that mechanical response soon disappears although the depolarization remains undiminished (Frank, 1958). From the results presented above, it has been concluded that the rate at which the mechanical response disappears is determined by the rate at which calcium ions leave the extracellular space when the muscle is placed in a calcium-free solution.

The latter conclusion is based on three lines of evidence. First, it was found that the rate at which the inhibition develops is determined by the dimensions of the muscle. Since the size of the corresponding muscle in different animals is determined by the number of cells in the muscle rather than the size of the cells, we should not expect to find a relation between muscle size and rate of development of inhibition if the rate was determined by the speed with which calcium ions left the intracellular space of the individual cells. In this respect it is interesting that average muscle fibre diameter often varies less in homologous muscles from animals of different species (Emerson & Emerson, 1959) than in different muscles from the same animal (Katz, 1948). Secondly, the acceleration in the rate of inhibition produced by the addition of a small amount of EDTA to the calcium-free solution shows that the speed of inhibition development is limited by the rate at which unbound calcium ions are removed from the extracellular spaces of the muscles. Finally, though our understanding of the cause of the inexcitability produced by placing a nerve or muscle in a calcium-free solution is incomplete (Edwards *et al.* 1956; Frankenhaeuser & Hodgkin, 1957; Frankenhaeuser, 1957; Ishiko & Sato, 1957), our knowledge of the processes involved in the generation of action potentials in these tissues (Hodgkin, 1951; Shanes, 1958*a*, *b*) leaves us with little reason to expect that calcium ions must be removed from the centre of the cells in order to prevent the production of an action potential. Therefore, the similarity in the time courses for the two effects (Fig. 10) suggests that the inhibition of contracture tension is dependent upon the rate at which calcium ions are lost from the extracellular space.

Niedergerke (1957) has presented evidence indicating that the loss of calcium ions from a strip of cardiac muscle bathing in a calcium-free solution is too slow to be accounted for solely on the basis of free diffusion out of the extracellular space. The free solution diffusion coefficient for calcium is $7{\cdot}8 \times 10^{-6}$ cm^2/sec (Wang, 1953), and the apparent diffusion coefficient, D', out of the extracellular space would be about 3 or 4 times

slower. because of obstruction by the fibres, if the loss of calcium from the muscle were determined by diffusion alone. Using the data available in Figs. 5 and 8, one can estimate the apparent diffusion coefficient D' for the loss of calcium ions when the toe muscle is placed in a calcium-free solution. Since the maximum tension of the contracture is inhibited by 50 % when the extracellular calcium concentration is reduced to 6 % of its initial value (Fig. 8), if the concentration at the surface had been held constant at 0 D' would be calculated by

$$D' = \frac{0{\cdot}372\,R^2}{t_{\frac{1}{2}}}.$$

By using values from Fig. 5 in this equation, one gets a value for D' of $1{\cdot}24 \times 10^{-6}$ cm^2/sec. By making a similar calculation based on the assumption of an undisturbed system (eqn. 1, p. 520), one obtains a value for D' of $13{\cdot}9 \times 10^{-6}$ cm^2/sec. Since the actual experimental conditions lay somewhere between these two theoretical extremes, the true value for D' is between the two calculated values. Such calculations are compatible with the assumption that the inhibition is determined by the free diffusion of calcium ions out of the extracellular spaces of the toe muscles.

There are at least two likely sites in the muscle where deprivation of calcium might account for the observed inhibition. If, as suggested here, calcium ions serve as a link between electrical and mechanical events in contraction, it would be sufficient to remove calcium ions from the outer surface of the muscle fibres in order to inhibit the mechanical response. On the other hand, it is conceivable that inhibition results from the removal of calcium from an intracellular location resulting in an inability of the contractile proteins to respond to their normal stimulus. The dependence of the time course of the inhibition on the rate at which calcium ions are removed from the extracellular spaces of the muscle provides strong suggestive evidence for the former possibility. Furthermore, production of unchanged caffeine contractures in a muscle bathed in a calcium-free solution (Fig. 11) renders the latter explanation of the inhibition unlikely.

The results presented above were easily and consistently obtained as long as described procedures were followed scrupulously. In this regard the possibility of contamination of the calcium-free solutions with slight quantities of calcium is worthy of special mention. This problem has been discussed in some detail by Frankenhaeuser (1957), but a few additional words are warranted. From the results presented in Fig. 8 we should expect that as little as 0·01 mM calcium in the calcium-free solution would prevent a complete elimination of the contracture, and in the one case that a toe muscle was equilibrated with this calcium concentration the contracture tension was only 76 % inhibited. This degree of contamination would be obtained if as little as 0·1 ml. of the primary solution clung to the sides of the bath and to the muscle when the solutions were changed. Such contamination can also occur unless special precautions are observed in preparing the water and in selecting the reagents for making the solutions.

Since Kuffler & Vaughan Williams (1953) have listed the extensor longus digiti IV as a muscle containing slow fibres, it is of interest to examine the evidence showing that the inhibition of the contracture produced by placing the toe muscle in a calcium-free solution is not limited to these slow fibres. It has been shown that the contracture produced by using isotonic potassium chloride, which activates all muscle fibres, can be eliminated by prior treatment of the muscle with a calcium-free solution (Fig. 2), and the time course of this inhibition is identical to that reported here when using 25 mM potassium, despite the considerably greater tensions developed during the isotonic potassium-chloride-induced contractures. Further, the isometric recording techniques used here would tend to favour the responses produced by twitch fibres and since the duration of the contractures produced by 25 mM potassium chloride rarely exceeded 60 sec, this would identify the response as resulting mainly from twitch fibre activation (Kuffler & Vaughan Williams, 1953). Finally, direct anatomical observation has shown that only 6–20 % of the fibres in the frog's toe muscle can be considered slow fibres (Gray, 1958), and it would seem most unlikely that this small number of fibres could be responsible for the tensions recorded in this work.

The concept of the role of calcium ions in contraction as proposed here has been vigorously supported in the writings of Heilbrunn (1943) and Sandow (1952). Some time ago Heilbrunn & Wiercinski (1947) found that calcium was the only physiologically occurring cation which would cause a shortening when injected into skeletal muscle fibres in low concentrations. Recently Bianchi & Shanes (1959) demonstrated an enhanced influx of calcium ions into frog sartorius muscle fibres during a twitch or potassium-induced contracture. Further, when the twitch was increased by replacing chloride ions with nitrate ions in the bathing solution, the extra influx of calcium ions per twitch was also increased.

A major criticism of the concept of the influx of calcium ions acting as a link between electrical and mechanical events in contraction of skeletal muscle has been voiced by Hill (1949). On the basis of his work showing that the contractile mechanisms is fully activated throughout the cross-section of the cell during the latent period, he has calculated that there is not sufficient time to permit a substance released at the surface of the cell to diffuse to its centre. Although theoretical models have been proposed to overcome this objection (Sandow, 1952; Shanes, 1958*b*), it need only be pointed out that this objection does not necessarily invalidate the role proposed here for the influx of calcium ions during contraction. It may be necessary for calcium ions only to reach the inner surface of the muscle membrane to initiate some other process which eventually leads to a mechanical response.

SUMMARY

1. The potassium-induced contracture of the extensor longus digiti IV muscle of the frog is reduced and eventually eliminated when the muscle is kept in a calcium-free solution for various periods of time before testing.

2. That the time course for this inhibition is determined by the rate that calcium ions leave the extracellular spaces of the muscle is shown by

(*a*) a relation between the speed with which the inhibition develops and the size of the muscle,

(*b*) increased speed of inhibition development by the addition of a small quantity of EDTA to the calcium-free solution, and

(*c*) a similar time course for the inhibition of the compound action potential of the same muscle when it is placed in a calcium-free solution.

3. Caffeine contractures can be obtained with the muscle in a calcium-free solution at a time when potassium-induced contractures have been completely eliminated, indicating that the contractile mechanism is still able to respond provided it receives an adequate stimulus.

4. These results, along with previous results showing that under the same conditions the potassium-induced depolarization is not reduced, support the idea that during an action potential or depolarization of a skeletal muscle fibre there is an influx of calcium ions which act as a link between the electrical and mechanical events in contraction.

I wish to thank Dr M. Nickerson and Dr I. Innes of this department and Andrew Huxley of the Department of Physiology, Cambridge University, for their helpful comments and suggestions and Mr B. Erickson for his competent technical assistance. I also wish to thank Dr H. R. Coish of the Department of Mathematical Physics for his assistance with the mathematics. The tubocurarine chloride was generously supplied by Burroughs Wellcome and Co. This work was supported by a grant from the National Research Council of Canada.

REFERENCES

Axelsson, J. & Thesleff, S. (1958). Activation of the contractile mechanism in striated muscle. *Acta physiol. scand.* **44**, 55–66.

Bianchi, C. P. & Shanes, A. M. (1959). Calcium influx in skeletal muscle at rest, during activity and during potassium contracture. *J. gen. Physiol.* **42**, 803–815.

Biedermann, W. (1896). *Electrophysiology*, Vol. 1. London: Macmillan.

Bülbring, E., Holman, M. & Lüllmann, H. (1956). Effects of calcium deficiency on striated muscle of the frog. *J. Physiol.* **133**, 101–117.

Chenoweth, M. B. (1956). Chelation as a mechanism of pharmacological action. *Pharmacol. Rev.* **8**, 57–87.

Crank, J. (1956). *The Mathematics of Diffusion.* Oxford: Clarendon Press.

Denton, E. J. (1948). The role of calcium in contractures of the rectus abdominis of the frog. *J. Physiol.* **107**, 32*P*.

EDWARDS, C., RITCHIE, J. M. & WILKIE, D. R. (1956). The effect of some cations on the active state of muscle. *J. Physiol.* **133**, 412–419.

EMERSON, J. D. & EMERSON, G. M. (1959). Comparison of gastrocnemius fiber diameters among mammalian species. *Fed. Proc.* **18**, 42.

FRANK, G. B. (1958). Inward movement of calcium as a link between electrical and mechanical events in contraction. *Nature, Lond.*, **182**, 1800–1801.

FRANKENHAEUSER, B. (1957). The effect of calcium on the myelinated nerve fibre. *J. Physiol.* **137**, 245–260.

FRANKENHAEUSER, B. & HODGKIN, A. L. (1957). The action of calcium on electrical properties of squid axons. *J. Physiol.* **137**, 218–244.

GASSER, H. S. (1930). Contractures of skeletal muscle. *Physiol. Rev.* **10**, 35–109.

GRAY, E. G. (1958). The structures of fast and slow muscle fibres in the frog. *J. Anat.* **92**, 559–562.

HAJDU, S. (1951). Observations on the temperature dependence of the tension developed by the frog muscle. *Arch. int. Physiol.* **59**, 58–61.

HEILBRUNN, L. V. (1943). *An Outline of General Physiology*, 2nd ed. pp. 535–539. Philadelphia: Saunders.

HEILBRUNN, L. V. & WIERCINSKI, F. J. (1947). The action of various cations on muscle protoplasm. *J. cell. comp. Physiol.* **29**, 15–32.

HILL, A. V. (1949). The abrupt transition from rest to activity in muscle. *Proc. Roy. Soc.* B, **136**, 399–420.

HODGKIN, A. L. (1951). The ionic basis of electrical activity in nerve and muscle. *Biol. Rev.* **26**, 339–409.

HODGKIN, A. L. & KEYNES, R. D. (1957). Movement of labelled calcium in squid giant axons. *J. Physiol.* **138**, 253–281.

ISHIKO, N. & SATO, M. (1957). The effect of calcium ions on electrical properties of striated muscle fibres. *Jap. J. Physiol.* **7**, 51–63.

KATZ, B. (1948). The electrical properties of the muscle fibre membrane. *Proc. Roy. Soc.* B, **135**, 506–534.

KUFFLER, S. W. (1946). The relation of electrical potential changes to contracture in skeletal muscle. *J. Neurophysiol.* **9**, 367–377.

KUFFLER, S. W. & VAUGHAN WILLIAMS, E. M. (1953). Properties of the 'slow' skeletal muscle fibres of the frog. *J. Physiol.* **121**, 318–340.

LECKIE, W. J. H. & TOMPSETT, S. L. (1958). The diagnostic and therapeutic use of edathamil calcium disodium (EDTA, Versene) in excessive inorganic lead absorption. *Quart. J. Med.* **27**, 65–82.

LÜTTGAU, H. C. & NIEDERGERKE, R. (1958). The antagonism between Ca and Na ions on the frog's heart. *J. Physiol.* **143**, 486–505.

MINES, G. R. (1913). On functional analysis by the action of electrolytes. *J. Physiol.* **46**, 188–235.

NIEDERGERKE, R. (1956*a*). The 'staircase' phenomenon and the action of calcium on the heart. *J. Physiol.* **134**, 569–583.

NIEDERGERKE, R. (1956*b*). The potassium chloride contracture of the heart and its modification by calcium. *J. Physiol.* **134**, 584–599.

NIEDERGERKE, R. (1957). The rate of action of calcium ions on the contraction of the heart. *J. Physiol.* **138**, 506–515.

NIEDERGERKE, R. & LÜTTGAU, H. C. (1957). Calcium and the contraction of the heart. Antagonism between calcium and sodium ions. *Nature, Lond.*, **179**, 1066–1067.

RINGER, S. (1883). A further contribution regarding the influence of the different constituents of blood on the contraction of the heart. *J. Physiol.* **4**, 29–42.

SANDOW, A. (1947). Latency relaxation and a theory of muscular mechano-chemical coupling. *Ann. N.Y. Acad. Sci.* **47**, 895–929.

SANDOW, A. (1952). Excitation-contraction coupling in muscular response. *Yale J. biol. Med.* **25**, 176–201.

SHANES, A. M. (1958*a*). Electrochemical aspects of physiological and pharmacological action in excitable cells. Part I. The resting cell and its alteration by extrinsic factors. *Pharmacol. Rev.* **10**, 59–164.

SHANES, A. M. (1958*b*). Electrochemical aspects of physiological and pharmacological action in excitable cells. Part II. The action potential and excitation. *Pharmacol. Rev.* **10**, 165–273.

STEN-KNUDSEN, O. (1954). The ineffectiveness of the 'window field' in the initiation of muscle contraction. *J. Physiol.* **125**, 396–404.

WANG, J. H. (1953). Tracer diffusion in liquids. IV. Self diffusion of calcium ion and chloride ion in aqueous calcium chloride solutions. *J. Amer. chem. Soc.* **75**, 1769–1772.

WARE, F. JR., BENNETT, A. L. & MCINTYRE, A. R. (1955). Cellular potentials in frog hearts. *Fed. Proc.* **14**, 158–159.

J. Physiol. (1960), **151**, *pp.* 539–550
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CIRCULATORY, RESPIRATORY AND METABOLIC RESPONSES TO ISOPROPYLNORADRENALINE IN MAN

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The effects of isopropylnoradrenaline on the heart and peripheral circulation in man were first studied by Barcroft & Konzett (1949). Since trace amounts of this amine have been reported in the adrenal glands of the cat, monkey and man (Lockett, 1954) and in the pulmonary venous blood of cats after stimulation of the cervical sympathetic chain (Lockett, 1957), a more detailed study has been made of its circulatory, respiratory and metabolic effects in man; a preliminary summary of the findings has already been reported (Cobbold, Galbraith, Ginsburg & Paton, 1958).

METHODS

The majority of experiments were performed on healthy adults; studies were also made in two patients after cervical sympathectomy and in one subject after lumbar sympathectomy. Subjects were tested at rest after a fast of 8–10 hr.

DL-Isopropylnoradrenaline (Isuprel, Winthrop) was infused intravenously in a dose of 0·1 μg/kg body weight/min for 30 min periods; the administration of the amine was preceded and followed by control infusions of sodium chloride solutions 0·9 % (w/v). Ascorbic acid 0·03 % was added to all solutions (Gaddum, Peart & Vogt, 1949).

Blood flow was recorded in hand, foot, forearm, or calf, at half-minute intervals by venous occlusion plethysmography (Barcroft & Swan, 1953). Blood pressure was measured at 2–5 min intervals by means of a sphygmomanometer; pulse rate was counted at the wrist. In some experiments arterial pressure and pulse rate were recorded continuously by a capacitance manometer system connected to a needle inserted into the brachial artery.

The rate of respiration was recorded by a chest pneumograph. Pulmonary ventilation was measured over successive 5 min periods by passing the expired air through low-resistance valves into a series of Douglas bags, or through a recording gas meter. In some subjects oxygen consumption was measured with the Benedict–Roth spirometer. In three subjects the pH of the arterial blood was estimated with the glass electrode, precautions being taken to prevent CO_2 loss from the blood.

Blood glucose was estimated by a modified Shaffer–Hartmann method (Haslewood & Strookman, 1939) in samples taken from the ear lobe or brachial artery. Lactic acid was measured by the method of Barker & Summerson (1941) in blood taken from a deep vein of the antecubital fossa.

In eighteen experiments isopropylnoradrenaline was infused into a brachial artery for 4–10 min, in doses ranging between 0·05 and 0·2 μg/min. Blood flow was measured in both hands in twelve of these infusions, and in both forearms in the remainder.

RESULTS

Intravenous isopropylnoradrenaline

Circulatory changes. Within 2 min of the start of the infusion heart rate and the blood pressure increased (Fig. 1). The mean rise in pulse rate was from 66 initially to 102 within 2 min; this rate was maintained throughout the infusion period, the mean rate at the end of the infusion being 108.

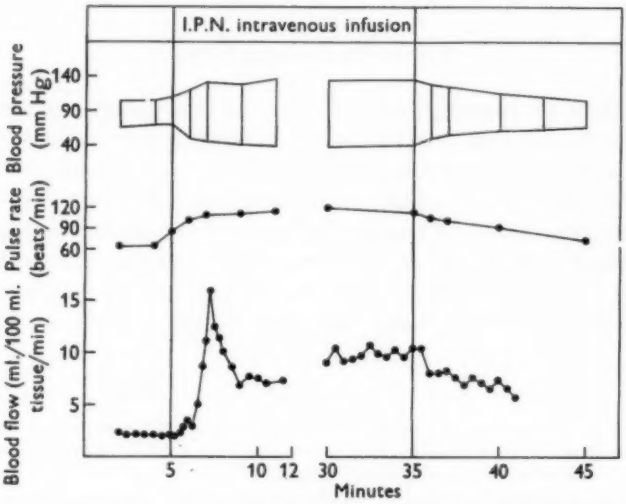

Fig. 1. The effect of intravenous infusion of isopropylnoradrenaline (I.P.N.) 0·1 μg/kg/min on the blood pressure, pulse rate and forearm blood flow; the period of infusion is indicated by the vertical lines. During the time between 12 and 30 min the blood pressure, pulse rate and blood flow were sustained at their increased values.

Initially there was a transient fall in both systolic and diastolic blood pressures coincident with the increase in heart rate (Fig. 2), lasting for some 10 sec; the pressure then rose from the resting average of 100/70 mm Hg to a mean of 120/45 mm Hg at 2 min, and 135/40 mm Hg 15 min after the beginning of the infusion. The pulse pressure was thus doubled.

There was a marked increase in forearm blood flow initially to a level about 700 % greater than the resting value; the flow then subsided rapidly to a level about 450 % above the control, and this was sustained throughout the period of infusion (Fig. 3, Table 1). Increases in calf blood flow were of a lesser degree, averaging 300 and 130 % above control values in the initial and sustained phases, respectively (Fig. 4, Table 1). There was a slight transient increase in hand or foot flow initially; thereafter the mean

flow was not greater than the resting flow (Figs. 3, 4, Table 2). During infusion the skin never became pale and in some subjects flushing of the face was observed. Similar changes in blood flow were recorded in sympathectomized limbs.

Forearm and calf blood flow remained high for at least 7 min after the infusion of isopropylnoradrenaline had ceased, the control level being gradually regained within 12–20 min. Heart rate and blood pressure

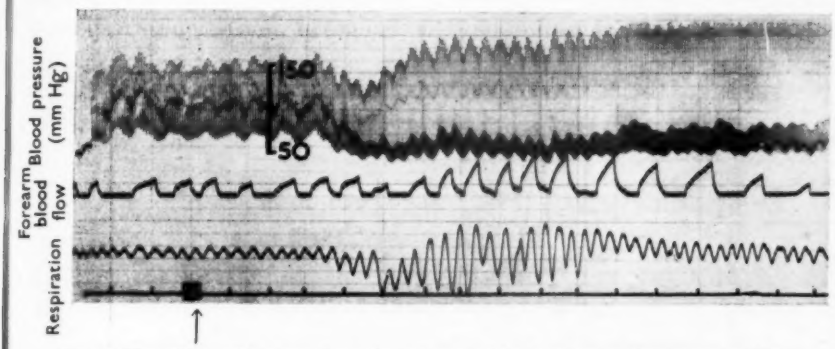

Fig. 2. The effect of isopropylnoradrenaline on blood pressure changes recorded from the brachial artery by capacitance manometer; forearm blood flow by plethysmograph and respiration from a chest stethograph. I.P.N. intravenous infusion 0·1 μg/kg/min commenced at arrow. Time marker, 0·25 min.

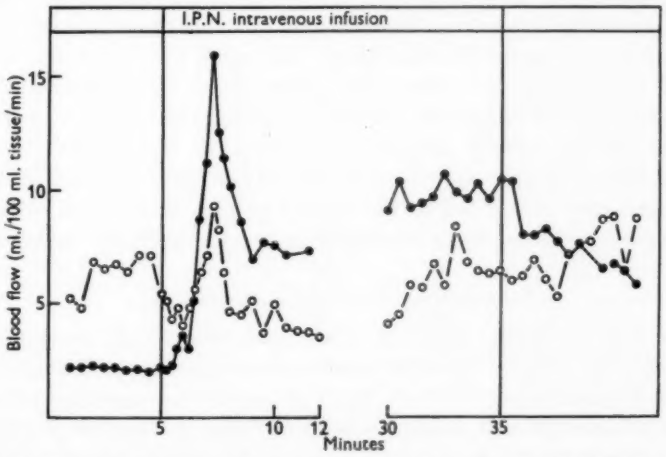

Fig. 3. Effect of intravenous infusion of isopropylnoradrenaline (I.P.N.) 0·1 μg/kg/min on forearm (●) and hand (○) blood flow. During the time between 12 and 30 min the flows were sustained at the levels shown before and after the break.

recovered more quickly, and were normal within 5 min of the end of the infusion.

Respiratory changes. Intravenous isopropylnoradrenaline had an immediate, powerful stimulatory effect upon respiration, the most marked action being upon the depth of breathing (Fig. 2). Pulmonary ventilation increased initially to about 66 % above the resting level and subsequently fell to a level of 30 % above the resting ventilation, which was maintained throughout the period of infusion of the amine. There was only a small increase in the rate of respiration. The changes in ventilation corresponded

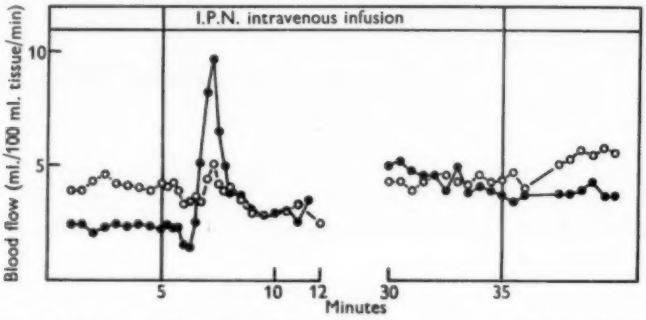

Fig. 4. Effect of intravenous infusion of isopropylnoradrenaline (I.P.N.) 0·1 μg/kg/min on calf (●) and foot (○) blood flow.

with an increase in oxygen consumption to an average of 30 % above the control level (Fig. 5). In the three subjects in whom arterial blood pH was measured this showed either no change or a small rise (Table 3).

Blood sugar and lactate. There was a slow rise in blood glucose concentration during the infusion, the average increase of 30 % above the fasting sugar level being reached after about 20 min (Fig. 6). The raised level of blood glucose was maintained for about 30 min after the end of the infusion, and then slowly fell to the control values. The lactate content of the venous blood remained unchanged during and after the infusion.

Intra-arterial isopropylnoradrenaline

There was no change in heart rate, blood pressure or venous lactate during intra-arterial infusions of isopropylnoradrenaline at different doses into the brachial artery. Blood flow in the forearm receiving the intra-arterial infusion showed changes similar to those recorded in the forearm during intravenous infusion (Fig. 7, Table 4). The mean increase in forearm flow was 350 % during the initial phase and 250 % during the sustained phase of activity. There was no change in flow in the contralateral uninfused forearm.

TABLE 1. Changes in forearm and calf blood flow during intravenous infusions of isopropylnoradrenaline (0·1 μg/kg/min) for 30-min periods

Subject	Blood flow (ml./min/100 ml. tissue) A	B	$B-A$	$\frac{B-A}{A}\times 100$
		Forearm		
C.P.	2·2	9·1	6·9	314
R.K.	2·0	4·6	2·6	130
A.C.	1·3	6·7	5·4	415
C.Q.	2·0	8·3	6·3	315
R.C.	3·0	15·3	12·3	410
H.G.	1·0	17·1	16·1	1610
I.D.	3·2	8·6	5·4	169
R.C.	2·4	6·3	3·9	163
J.D.	2·0	13·7	11·7	585
Mean	2·1	10·0	7·8	457
		Calf		
J.P.	3·3	4·8	1·5	45
J.G.	1·5	2·2	0·7	47
P.M.	5·5	7·9	2·4	44
E.G.	2·7	5·5	2·8	104
M.M.	1·3	5·4	4·1	315
H.B.	1·7	2·3	0·6	35
J.C.	1·7	3·6	1·9	112
M.M.	1·0	5·9	4·9	490
H.B.	2·0	3·2	1·2	60
M.D.	2·3	4·1	1·8	78
Mean	2·3	4·5	2·2	133

A, mean flow during the 4 min before infusion; *B*, mean flow during the last 4 min of the infusion.

TABLE 2. Changes in hand and foot blood flow during intravenous infusion of isopropylnoradrenaline (0·1 μg/kg/min) for 30-min periods

Subject	Blood flow (ml./min/100 ml. tissue) A	B	$B-A$	$\frac{B-A}{A}\times 100$
		Hand		
C.P.	8·1	12·9	+4·8	+ 59
R.K.	7·8	2·4	−5·4	− 69
A.C.	3·7	1·7	−2·0	− 54
C.Q.	6·1	5·3	−0·8	− 13
R.C.	7·2	10·7	+3·5	+ 49
H.G.	2·9	4·4	+1·5	+ 52
J.C.	9·0	2·8	−6·2	− 69
M.M.	5·0	4·0	−1·0	− 20
H.B.	6·3	9·1	+2·8	+ 44
M.D.	8·1	8·9	+0·8	+ 10
Mean	6·4	6·2	−0·2	− 11
		Foot		
J.G.	7·3	5·9	−1·4	− 19
J.P.	4·6	4·4	−0·2	− 4
P.B.	3·7	9·7	+6·0	+162
E.G.	3·5	3·0	−0·5	− 14
M.M.	2·1	1·0	−1·0	− 48
H.B.	5·7	4·7	−1·0	− 18
M.B.	4·3	4·5	+0·2	+ 5
J.C.	2·3	1·6	−0·7	− 30
Mean	4·2	4·3	+0·2	+ 4

A, mean flow during the 4 min before infusion; *B*, mean flow during the last 4 min of the infusion.

Blood flow in the infused hand was unchanged initially and during short periods of infusion; there was, however, sometimes a slight increase in flow towards the end of intra-arterial infusions lasting 10 min.

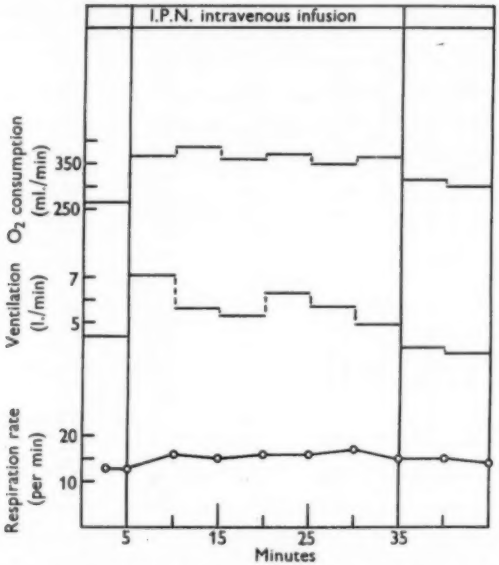

Fig. 5. Effect of intravenous infusion of isopropylnoradrenaline (I.P.N.) 0·1 μg/kg/min on the rate of respiration, pulmonary ventilation and oxygen consumption.

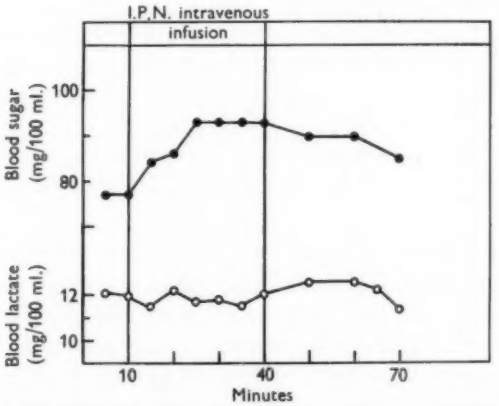

Fig. 6. Effect of intravenous infusion of isopropylnoradrenaline (I.P.N.) 0·1 μg/kg/min on the blood sugar (●) and blood lactate (○).

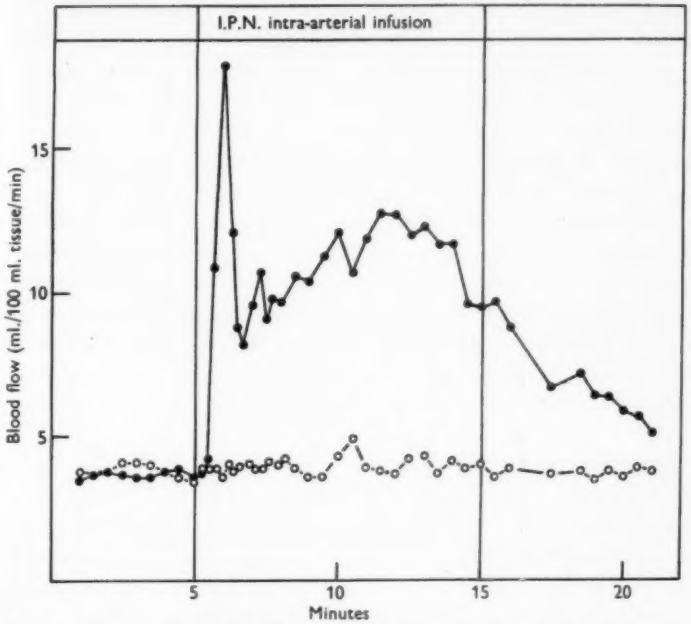

Fig. 7. Effect of intra-arterial infusion of isopropylnoradrenaline (I.P.N.) (0·05–0·2 μg/min) on forearm blood flow. ●, flow in the arm infused; ○, contralateral, uninfused, forearm. The points represent mean values for the three subjects of Table 4.

TABLE 3. Arterial blood pH during intravenous infusion of isopropylnoradrenaline (0·1 μg/kg/min)

Subject	Control	3 min after start of infusion	9 min after start	10 min after end of infusion
M.D.	7·28	—	7·33	7·32
J.P.	7·33	7·38	7·40	7·38
J.G.	7·44	7·43	7·42	7·37

TABLE 4. Changes in forearm blood flow during intra-arterial infusions of isopropylnoradrenaline for 10 min periods

		Blood flow (ml./min/100 ml. tissue)				
Subject	Dose (μg/min)	*A*	*B*	*a*	*b*	$\frac{B-E}{E} \times 100$
J.P.	0·05	5·4	11·4	3·5	3·4	119
	0·1	4·0	9·8	3·6	3·3	165
	0·2	2·6	22·5	4·6	4·7	733
J.C.	0·05	2·4	5·7	4·3	4·7	119
	0·1	3·7	6·2	3·1	2·8	88
H.J.	0·1	4·0	15·8	4·0	5·9	164
	Mean	3·7	11·9	3·9	4·1	231

A, mean flow in test forearm during the 4 min before infusion; *B*, mean flow in test forearm during the last 4 min of infusion; *a*, mean flow in contralateral control forearm during the 4 min before infusion; *b*, mean flow in contralateral forearm during the last 4 min of the infusion; *E*, Ab/a.

DISCUSSION

The circulatory changes which have been observed during the administration of isopropylnoradrenaline are in agreement with those reported by Barcroft & Konzett (1949); the longer period of infusion used in the present study showed that the effects of the amine persist throughout the infusion and for a short time afterwards.

The increased flow in forearm and calf is probably due in the main to vasodilatation in the blood vessels of skeletal muscle. Direct measurement of blood flow in hind-limb muscles of the dog has shown marked dilatation after the administration of isopropylnoradrenaline (Green, Shearin, Jackson, Keach & Denison, 1954). The fact that dilatation occurred after intravenous and direct intra-arterial infusion, and in healthy and sympathectomized subjects, showed further that it was a direct effect of the drug.

The vasodilator action of intravenous isopropylnoradrenaline in forearm and calf resembles that of adrenaline—an initial large increase in flow and a subsequent secondary phase of dilatation which is sustained throughout the infusion (Whelan, 1952; Barcroft & Swan, 1953). Isopropylnoradrenaline is, however, much the more powerful dilator, causing sustained dilatation even after intra-arterial administration, whereas comparable doses of adrenaline produce only transient increases in flow (Barcroft & Swan, 1953). The action of isopropylnoradrenaline contrasts strikingly with that of noradrenaline, which has a marked constrictor effect on the blood vessels of muscle (Barcroft & Konzett, 1949; Cobbold & Vass, 1953).

Whilst both adrenaline and noradrenaline have a powerful constrictor action on the vessels of the hand and foot, isopropylnoradrenaline caused weak but transient vasodilatation. During prolonged intra-arterial infusions dilatation was also observed, but whether this represents an action on skin blood vessels, similar to that recorded in dogs when blood flow was measured directly in the saphenous artery (Walters, Cooper, Denison & Green, 1955), or dilatation in muscle, is not clear.

As a cardiac stimulant isopropylnoradrenaline is more powerful than adrenaline, the tachycardia caused by adrenaline being marked only in the first few minutes of intravenous infusion, whereas that with isopropylnoradrenaline continued throughout. Our subjects, all of whom had previously received infusions of adrenaline, always remarked upon this difference and on the prolonged and powerful cardiac action which occasionally led to some degree of apprehension. This stimulating effect of isopropylnoradrenaline is in marked contrast to that of noradrenaline, which causes a transient bradycardia of reflex origin. Lands & Howard (1952) found that isopropylnoradrenaline was more effective than either adrenaline or nor-

adrenaline in increasing the rate and amplitude of contraction of the perfused heart.

The effect of isopropylnoradrenaline on blood pressure is also marked and prolonged. The increase in systolic pressure accompanies, and appears to be due to, the increase in rate and force of the heart beat. This phenomenon is also seen with intravenous infusions of adrenaline and noradrenaline, but whereas noradrenaline causes a rise in the diastolic pressure, both adrenaline and isopropylnoradrenaline produce a fall, the effect being much greater with the latter. The fall in diastolic pressure can, in part, be accounted for by the dilatation in muscle blood vessels. It is unlikely, however, that this can be wholly responsible for the marked fall in pressure and additional dilatation must occur elsewhere. A probable site is in the splanchnic bed, and it is interesting that Green, Deal, Bardhanabaedya & Denison (1955), who measured blood flow directly in the mesenteric artery of the dog, reported a large increase in flow after isopropylnoradrenaline. The renal blood flow may also increase (Aviado, Wnuck & de Beer, 1958).

The effect of isopropylnoradrenaline on respiratory activity resembles that described for adrenaline and noradrenaline by Whelan & Young (1953), an abrupt increase in pulmonary ventilation accompanied by a small change only in the rate of breathing. The stimulating action of isopropylnoradrenaline is, however, more powerful and persists throughout the infusion, whereas the effect of adrenaline or noradrenaline decreases during the period of infusion. Oxygen consumption increased along with the increase in pulmonary ventilation, and isopropylnoradrenaline is as potent as adrenaline in this respect. The calorigenic action is accompanied by a slow rise in blood glucose level but less than that observed with adrenaline (Bearn, Billing & Sherlock, 1951).

Since there was no change in lactate concentration it is not possible to attribute the vasodilator effects or the stimulation of respiration to a release of lactic acid. This contrasts with the rise in venous lactate observed during the administration of adrenaline (Bearn *et al.* 1951; Barcroft & Cobbold, 1956). The fact that there was no fall in arterial pH after infusing isopropylnoradrenaline suggests further that other acid metabolites can be excluded. The small rise in arterial pH could be attributed to over-ventilation, as was suggested for adrenaline (Bradley, Gaskell, Holland, Lee & Young, 1954).

Thus in general, the effects of isopropylnoradrenaline resemble those of adrenaline more than noradrenaline. The actions of the three related amines in comparable doses (0·1 μg/kg/min) in man are summarized in Table 5.

In the present study marked effects were obtained with the racemic

form, DL-isopropylnoradrenaline. The laevo isomer, however, which would be the compound expected to occur naturally, is even more powerful and is effective at very low concentrations (Lands, Luduena & Tullar, 1954).

The powerful action on the heart and respiration, and the accompanying marked vasodilatation, would make for a highly effective physiological agent, if isopropylnoradrenaline were normally released in the body. That this may occur was suggested by Lands & Tainter (1953), who speculated on the possibility that a substance like isopropylnoradrenaline might normally be formed to reinforce the effects of adrenaline in organs where

TABLE 5

	L-Noradrenaline	L-Adrenaline	DL-Isopropyl-noradrenaline
Muscle blood flow	Decrease	Increase	Large increase
Skin blood flow	Decrease	Decrease	Small increase
Heart rate	Decrease	Transient increase	Sustained increase
Systolic pressure	Rise	Rise	Marked rise
Diastolic pressure	Rise	Fall	Marked fall
O_2 consumption	Small increase	Increase	Increase
Pulmonary ventilation	Small increase	Increase	Large increase
Blood sugar	Small increase	Increase	Moderate increase
Blood lactate	No change	Increase	No change

sympathetic inhibitory action is dominant. The findings of Lockett (1954, 1957) in cats would support such an assumption. The effectiveness of even minute concentrations of isopropylnoradrenaline in stimulating the rate and amplitude of contraction of the isolated auricle led Garb, Penna & Ganz (1956) to suggest that small amounts of the amine could be of physiological importance. Walters *et al.* (1955) also suggested that the occurrence of both vasoconstrictor and vasodilator effects with adrenaline accords with it behaving as a mixture of noradrenaline and isopropylnoradrenaline, the action of the former being dominant before treatment with adrenergic blocking drugs, while isopropylnoradrenaline predominates after the administration of such drugs. Furthermore, the sustained vasodilatation seen in human muscle during intravenous infusions of adrenaline has been attributed to the release of some unidentified dilator compound (Whelan, 1952), and it has also been suggested that the stimulating effect of adrenaline and noradrenaline on respiration might be due to their conversion to a related substance (Young, 1957). It is tempting to assume that isopropylnoradrenaline plays a part in these responses, but no definite evidence yet exists.

The observation that infusions of isopropylnoradrenaline reproduced the normal left ventricular response to exercise in dogs more accurately than did adrenaline or noradrenaline (Rushmer & West, 1957; Rushmer & Smith, 1959) raises, also, the possibility that it is released during exercise.

The involvement of isopropylnoradrenaline in physiological responses must however remain speculative until unequivocal evidence has been provided of its release in man in such circumstances.

SUMMARY

1. Intravenous infusion of DL-isopropylnoradrenaline (0·1 μg/kg/min) in man increases the heart rate and systolic blood pressure and causes a fall in diastolic blood pressure. Blood flow in forearm and calf shows a marked initial transient increase followed by a lesser but sustained increase. Blood flow in hand and foot shows an initial, small and transient increase only. There is a small increase in respiratory rate and a sustained increase in the depth of breathing; oxygen consumption increases. Blood sugar rises slowly; there is no change in blood lactate. There is no difference between the response in normal and sympathectomized limbs.

2. Intra-arterial infusion of isopropylnoradrenaline (0·05–0·2 μg/min) causes the same change in blood flow in the infused limb as that recorded after intravenous administration, but with no systemic effect.

3. The response to isopropylnoradrenaline is discussed and compared with that to adrenaline and noradrenaline.

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REFERENCES

AVIADO, D. M. JR., WNUCK, A. L. & DE BEER, E. J. (1958). The effects of sympathomimetic drugs on renal vessels. *J. Pharmacol.* **124**, 238–244.

BARCROFT, H. & COBBOLD, A. F. (1956). The action of adrenaline on muscle blood flow and blood lactate in man. *J. Physiol.* **132**, 372–378.

BARCROFT, H. & KONZETT, H. (1949). On the actions of noradrenaline, adrenaline and isopropyl noradrenaline on the arterial blood pressure, heart rate and muscle blood flow in man. *J. Physiol.* **110**, 194–204.

BARCROFT, H. & SWAN, H. J. C. (1953). *Sympathetic Control of Human Blood Vessels.* London: Edward Arnold and Co.

BARKER, S. B. & SUMMERSON, W. H. (1941). The colorimetric determination of lactic acid in biological material. *J. biol. Chem.* **138**, 535–554.

BEARN, A. G., BILLING, B. & SHERLOCK, S. (1951). The effect of adrenaline and noradrenaline on hepatic blood flow and splanchnic carbohydrate metabolism in man. *J. Physiol.* **115**, 430–441.

BRADLEY, R. D., GASKELL, P., HOLLAND, W. W., LEE, J. DE G. & YOUNG, I. M. (1954). The acid–base changes in arterial blood during adrenaline hyperpnoea in man. *J. Physiol.* **124**, 213–218.

COBBOLD, A. F., GALBRAITH, H. J. B., GINSBURG, J. & PATON, A. (1958). Circulatory, metabolic and respiratory effects of *iso*propylnoradrenaline in man. *J. Physiol.* **141**, 40*P*.

COBBOLD, A. F. & VASS, C. C. N. (1953). Responses of muscle blood vessels to intra-arterially and intravenously administered noradrenaline. *J. Physiol.* **120**, 105–114.

GADDUM, J. H., PEART, W. S. & VOGT, M. (1949). The estimation of adrenaline and allied substances in blood. *J. Physiol.* **108**, 467–481.

GARB, S., PENNA, M. & GANZ, A. (1956). Effects of epinephrine, norepinephrine and isopropylarterenol on the isolated auricles of four mammalian species. *Amer. J. Physiol.* **185**, 332–336.

GREEN, H. D., DEAL, C. P. JR., BARDHANABAEDYA, S. & DENISON, A. B. JR. (1955). The effects of adrenergic substances and ischemia on the blood flow and peripheral resistance of the canine mesenteric vascular bed before and during adrenergic blockade. *J. Pharmacol.* **113**, 115–123.

GREEN, H. D., SHEARIN, W. T. JR., JACKSON, T. W., KEACH, L. M. & DENISON, A. B. JR. (1954). Isopropylnorepinephrine blockade of epinephrine reversal. *Amer. J. Physiol.* **179**, 287–294.

HASLEWOOD, G. A. D. & STROOKMAN, T. A. (1939). A method for the estimation of 'true' sugar in 0·05 ml. of blood. *Biochem. J.* **33**, 920–923.

LANDS, A. M. & HOWARD, J. W. (1952). A comparative study of the effects of L-arterenol, epinephrine and isopropylarterenol on the heart. *J. Pharmacol.* **106**, 65–76.

LANDS, A. M., LUDUENA, F. P. & TULLAR, B. F. (1954). The pharmacologic activity of the optical isomers of isopropylarterenol (isuprel) compared with that of the optically inactive analog (1-3,4-dihydroxyphenyl)-2-isopropylamino ethane HCl. *J. Pharmacol.* **111**, 469–474.

LANDS, A. M. & TAINTER, M. L. (1953). The effect of changes in molecular configuration on inhibitory sympathomimetic action. *Arch. exp. Path. Pharmak.* **219**, 76–90.

LOCKETT, M. F. (1954). Identification of an isoprenaline-like substance in extracts of adrenal glands. *Brit. J. Pharmacol.* **9**, 498–505.

LOCKETT, M. F. (1957). The transmitter released by stimulation of the bronchial sympathetic nerves of cats. *Brit. J. Pharmacol.* **12**, 86–96.

RUSHMER, R. F. & SMITH, O. A. JR. (1959). Cardiac control. *Physiol. Rev.* **39**, 41–68.

RUSHMER, R. F. & WEST, T. C. (1957). Role of autonomic hormones on left ventricular performance continuously analysed by electronic computers. *Circulation Res.* **5**, 240–246.

WALTERS, P. A. JR., COOPER, T. W., DENISON, A. B. JR. & GREEN, H. D. (1955). Dilator responses to isoproterenol in cutaneous and skeletal muscle vascular beds; effects of adrenergic blocking drugs. *J. Pharmacol.* **115**, 323–328.

WHELAN, R. F. (1952). Vasodilatation in human skeletal muscle during adrenaline infusions. *J. Physiol.* **118**, 575–587.

WHELAN, R. F. & YOUNG, I. M. (1953). The effect of adrenaline and noradrenaline infusions on respiration in man. *Brit. J. Pharmacol.* **8**, 98–102.

YOUNG, I. M. (1957). Some observations on the mechanism of adrenaline hyperpnoea. *J. Physiol.* **137**, 374–395.

J. Physiol. (1960), **151**, *pp.* 551–565
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THE RESPONSE OF THE NEW-BORN BABY TO INFLATION OF THE LUNGS

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It has been shown by Cross, Dawes & Mott (1959) that the output of the left heart in the new-born lamb may increase about threefold at the time of birth, but does not further increase with anoxia after birth. We thought in view of this that it would be of interest to measure the cardiac output of the new-born infant. The nitrous oxide method of measuring pulmonary capillary blood flow described by Lee & DuBois (1955) seemed suitable, as blood samples are not required. The first step, however, in this technique is to ask the conscious, co-operative adult to take a deep breath and to hold it for 10 sec. We therefore sought ways to 'ask' the new-born baby to take a deep breath and hold it. The first technique tried was inflation of the lungs of the sleeping infant. We became so interested in the reflex results consequent upon lung inflation that we are reporting here our observations. Some of these have already been reported briefly (Cross, Klaus, Tooley & Weisser, 1959*a*, *b*).

METHODS

Initially we watched the response of a sleeping infant to inflation of the lungs achieved by compression of a rubber bag connected to an infant face mask (as used by anaesthetists). As the infant continued to sleep and accepted inflation quietly, it was decided to investigate the response quantitatively. A body plethysmograph similar to the instrument used by Cross (1949) was constructed, differing only in that its volume (15·5 l.) is smaller than that originally described, and that the lid has a neat positive-pressure seal which allows almost instantaneous release of the infant should it seem desirable. The small size of the plethysmograph is advantageous in giving easily measured pressure changes with each breath of the infant. The pressure changes in the plethysmograph were converted to the volumes of each breath. (Corrections have to be applied for the size of the infant.) Pressure changes in the box relative to atmospheric pressure were measured with a sensitive differential Statham strain gauge and recorded by pens with a Grass amplifier and recorder. A flat response was found in the system up to about 7 c/s.

Method of inflation. Two methods of inflation were used. In the first, a positive pressure was applied through the mask to the face of the infant. A small Foregger anaesthetic face

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mask (dead space, 32 ml.) was held firmly on the face of the infant, with soft paraffin to minimize leaks when pressure was applied. However, some leak was almost always present; for this reason it was necessary to apply a pressure that could remain constant in spite of the leak, and so a weighted 6 l. spirometer was used as the source of the pressure and at the time of inflation was opened to the mask. A water manometer in the spirometer monitored the pressure applied; a strain gauge in the mask recorded the actual pressure at the mouth.

The second method of inflation of the lungs was by decompression of the body of the infant in the plethysmograph. A 20 l. bottle was decompressed to a known pressure, and when it was opened to the plethysmograph through 1 in. (2·5 cm) tubing and a tap, an equilibration pressure was rapidly applied to the infant's body (about 0·03 sec). The advantage of positive-pressure inflation with the mask was that the volume change of the infant's body could be recorded, but the mask caused not only restlessness but over-ventilation owing to the dead-space effects. To try to minimize these the mask was ventilated in between experimental applications of the pressure by a flow of gas from a compressed-air cylinder. With decompression of the body no mask was necessary, and a quietly sleeping infant was more easily obtained, but the increase in the volume of the baby's body could no longer be measured with any accuracy. A saline-filled oesophageal tube and Statham gauge (response time, 7 c/s) recorded the changes of pressure in the oesophagus, and was used as a measure of the intrapleural pressure (Dornhorst & Leathart, 1952). On certain occasions an air-filled, soft-walled rubber balloon was attached to the oesophageal tube, and this was used for recording oesophageal pressure. This system should have the advantage, of course, that absolute pressures can be recorded; when a saline-filled catheter is used, and the precise height of the open end is not known in relation to the manometer head, only *changes* of pressure can be measured. The disadvantage of the air-filled balloon is that it is a great deal more difficult to pass into the infant and causes much more disturbance, so that frequently a resting infant was not obtained. Even with a saline-filled catheter it is apparent that this does not always give a faithful record of changes in intrapleural pressure, in spite of frequent flushings. Thus, with successive inflations an identical change of volume in the baby might occur each time, indicating that it had taken a large gasp, yet the oesophageal pressure change at the one inflation might show no negative-pressure swing to account for the increase in volume but the next recording would show a large deflexion. Artifacts of this type were fortunately not common.

Experimental technique

When the mother had given permission for the study, on most occasions a No. 8 (French) polythene feeding tube was passed through the infant's nose, about 15 cm past the nares. This was filled with physiological saline solution, and the fact that it was in the thoracic oesophagus was checked by seeing that the pressure swing was negative with inspiration.

The infant was then placed in the plethysmograph and sealed in with the rubber cuff (made by Dunlop Rubber Co.; Cross, 1949) around the face. Checks for leaks were applied by adding a known volume of air to the plethysmograph and noting whether the base-line deflexion of pressure within the plethysmograph was maintained. If the infant slept quietly, then different inflation pressures were applied, and the results recorded. Any particular infant was given only one type of inflation, for the system was too complex to change in the time available for study. As most infants had a mask over the face, the respiratory rate was higher than would be expected in the resting state.

RESULTS

One hundred and thirty attempts were made on ninety-five babies. Thirty infants between the ages of 1 hr and 124 hr were studied successfully on forty-three different occasions, thirty-eight of these by positive-

pressure inflation through a mask, and five by body decompression. There was one premature (about 34 weeks gestation) infant of 8 days of age. On thirty-one occasions the observations were made on babies with oesophageal tubes; twelve were without. The results reported here are concerned chiefly with two types of respiratory reflex which may result from lung inflation. The first is the apnoea induced by inflation (Hering-Breuer reflex); the other is the gasp which may follow on inflation. Figure 1 illustrates both effects in successive inflations in one baby.

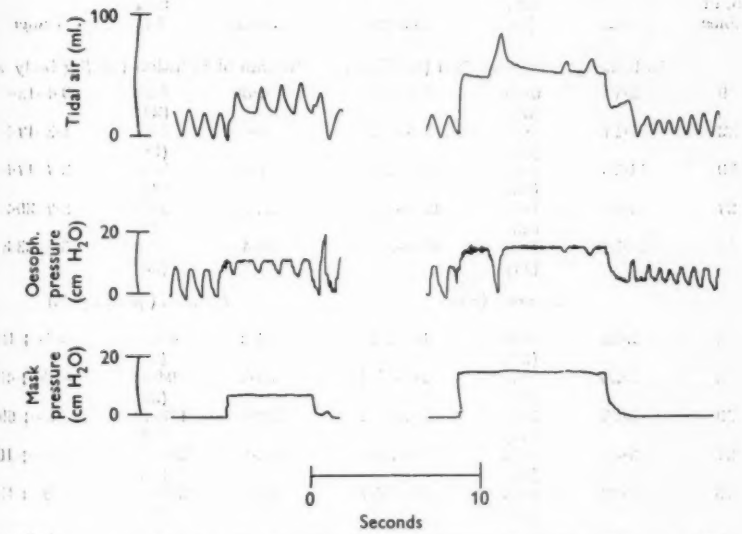

Fig. 1. Two successive inflations of baby No. 87, male, 3·9 kg, age 66 hr. From above downwards, tidal air (inspiration upwards), oesophageal pressure and mask pressure. In all diagrams positive pressure change is recorded as an upward deflexion. In the first inflation a pressure of 8 cm H_2O was applied and the baby showed some increase in volume and slight increase in expiratory pause. In the second inflation (pressure 16 cm H_2O) apnoea was much more pronounced and an inspiratory gasp occurred after some initial apnoea.

The apnoeic response

Table 1 shows the effect of various inflation pressures on the apnoea and the change in volume of the baby. The longest period of apnoea after inflation was recorded, both as the actual number of seconds of apnoea, and as a percentage of the normal mean respiratory cycle for the last ten breaths before the inflation. (Thus no change in rate will score 0% and 1 cycle missed = 100%.) An attempt was made to make the inflations during the expiratory pause, but this was difficult in some of the babies

who were breathing rapidly. The longest apnoeic period that occurred after the inflation was chosen for measurement. Figure 1 shows records obtained from a baby who had been inflated, and after an initial short apnoea there was a gasp, and the subsequent apnoea, which was at a greater volume, was more prolonged. Almost certainly the gasp had an effect on the new volume of inflation which the baby reached and on the length of the apnoea, but it was not seen how to separate these factors.

TABLE 1

No. of babies	Mean	S.D. (*n*)	Range	Mean	S.D. (*n*)	Range
	Inflation pressure applied (cm H_2O)			Volume of inflation (ml./kg body wt.)		
9	3·73	0·89 (29)	1·4–4·9	4·80	4·58 (24)	0·4–13·0
32	7·17	1·51 (83)	5·0–9·9	6·49	4·17 (69)	1·3–17·3
30	11·93	1·42 (82)	10·0–14·9	7·69	3·26 (74)	2·7–17·0
27	17·98	1·18 (82)	15·0–19·9	12·33	3·15 (67)	5·7–20·2
16	21·88	1·47 (47)	20·0–25·4	14·41	3·78 (38)	6·8–23·8
	Apnoea (sec)			Apnoea (% of cycle)		
9	2·99	1·74 (29)	1·2–7·0	54·2	48·97 (29)	−3–+150
32	3·26	2·64 (83)	1·0–13·8	81·2	94·53 (83)	−6–+400
30	3·66	3·23 (82)	1·2–20·0	127·0	128·72 (82)	0–+590
27	7·48	5·58 (82)	1·0–27·0	342·1	259·19 (82)	−35–+1070
16	6·62	4·48 (47)	1·3–18·7	309·1	264·16 (47)	0–+1350

The results are grouped here according to the effective pressure applied to the baby; the effective pressure is the difference between the pressure at the mouth and the pressure on the body wall. Apnoea is expressed in terms of the length of time occupied by the respiratory cycle following inflation; alternatively as the percentage of the normal mean respiratory cycle for the last ten breaths before the inflation.

The volume of inflation has been taken as being the change of volume from the resting expiratory position of the baby to the new resting expiratory position; once again, the intervention of a gasp may make this volume difficult to read, but in general it is correct to say that the baby, when it next breathes, will take an inspiration from the new inflated volume. In Table 1 it will be observed that there is a large variation in the volume of inflation of the baby at different pressures, and that the length of apnoea again is exceedingly variable. It is worth while to study the ranges which have been recorded, as well as the means. If one observes the means, there is in fact a consistent increase in the volume of the baby with increasing inflation pressures, and there is also a tendency for the length of apnoea to

increase with volume. These over-all results, while they give some picture of the observations made and the variants of them, very considerably obscure the changes in individual babies, and we thought it important to study the changes of the individual more closely. For the purpose of this study, twenty studies (on eighteen babies) were selected from the original forty-three observations, on two grounds—one, there were more than six

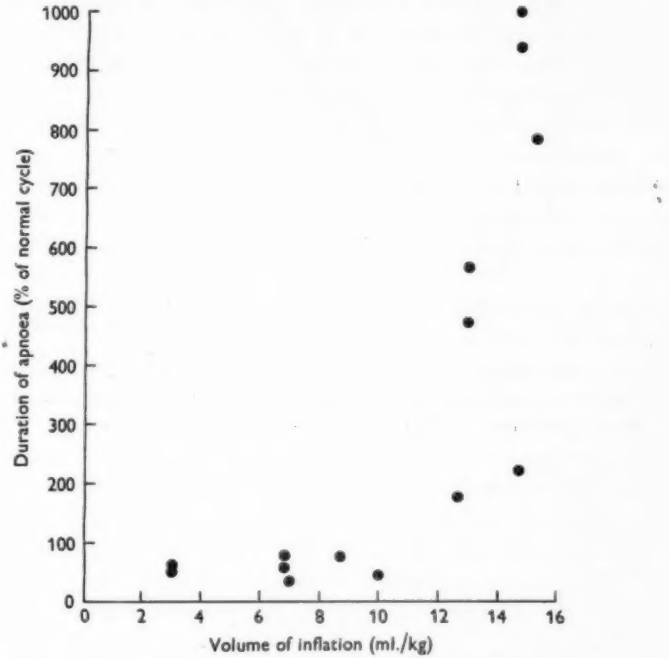

Fig. 2. Plot of duration of apnoea (expressed as percentage of normal cycle) against the volume of inflation. Baby No. 105, male, 3 kg, age 42 hr. It will be observed that there is a curvilinear relationship between the volume of inflation and the duration of apnoea.

inflations in one study, and secondly, that these inflations had been in not less than three of the pressure groups shown in Table 1. These twenty experiments (in which 75% of the total number of inflations had been performed) were examined to find what relationship there might be between pressure, volume and duration of apnoea.

It was found, by inspection of the plots from these babies, that the most consistent apparent correlation was a curvilinear relationship between the volume and the duration of apnoea of the baby. Figure 2 shows this result in one baby. It will be observed that with increasing volume of inflation

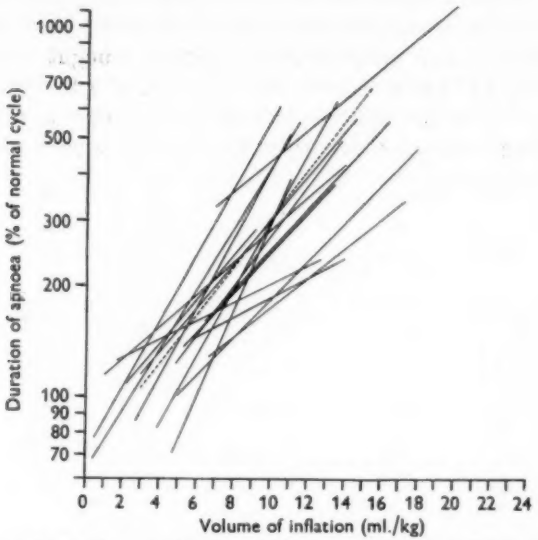

Fig. 3. The calculated regression line for duration of apnoea (log. scale) plotted against the volume of inflation for the seventeen babies mentioned in the text. The individual baby of Fig. 2 is represented by a dotted line.

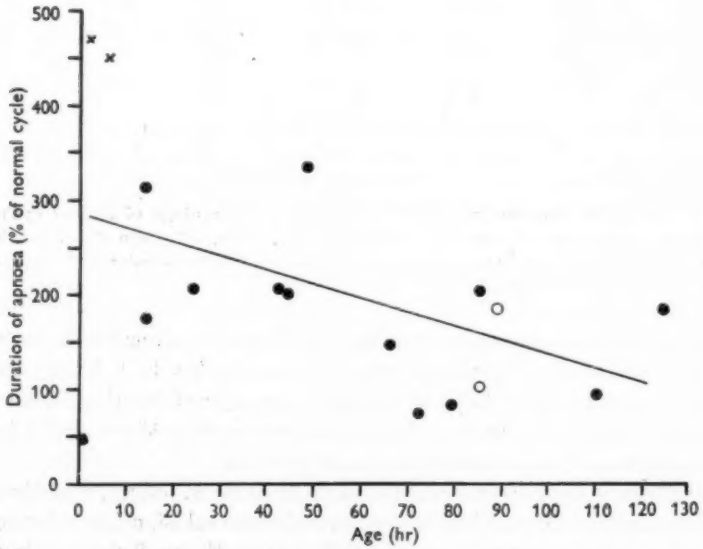

Fig. 4. Duration of apnoea plotted against the age in hours for an inflation of 10 ml./kg: ●, results from thirteen different babies; × and ○, results from one baby examined on two occasions.

the duration of apnoea goes up disproportionately. In order to test whether there was any consistency of this relationship in the twenty babies chosen for study, we have examined, by the method of least squares, the correlation between the logarithm of the duration of apnoea compared with the volume of inflation per kilogram of baby. It was found that of these twenty studies, seventeen showed a significant correlation coefficient at levels of $< 0{\cdot}05$, ten of them being significant at the $< 0{\cdot}001$ level. The three babies who did not show statistical significance did, however, show a correlation coefficient significant at the level of $< 0{\cdot}1$. It would seem therefore that this curvilinear relationship between duration of apnoea and volume genuinely exists, although we would not seek by this method of expression to give a more precise definition of this relationship.

In Fig. 3 the calculated regression lines for the seventeen significant studies are shown; the dotted line is the regression line relating to baby No. 105 (shown in Fig. 2). It will be seen that there is a family of curves which have fairly similar slopes.

From these lines it was possible to examine one other aspect of the effect of inflation. It had been observed that, in some babies who had been examined more than once, the duration of apnoea diminished with age, and for this reason the effect of age upon the duration of apnoea was investigated. This had to be done at a fixed inflation, and for this purpose the volume of 10 ml./kg was chosen, and the calculated length of apnoea at this time for each baby was read off from the regression line in Fig. 3. In Fig. 4 the plot of these results is shown, and although there is considerable scatter it appears from these seventeen results from fifteen babies that the length of apnoea does diminish with increasing age over the rather short period during which the babies were available to us ($r = 0{\cdot}500$, $P < 0{\cdot}05$, $n = 17$).

The gasp

A gasp in response to inflation was common in the baby. A gasp was said to have occurred when the oesophageal pressure swing was greater and of shorter duration than a normal inspiration. With higher pressures the gasp tended to occur early, as in Fig. 5. Figure 6 shows the details of an inflation giving rise to a gasp on an extended time scale. It will be noted that the gasp occurred half a second after the inflation pressure was applied, when passive expansion of the lungs was almost complete. The onset of the gasp may be more delayed, as in the second inflation in Fig. 1, and one baby may show both an immediate and a delayed gasp when inflated. In the younger babies, who gasped more readily, the gasp was not necessarily related to apnoea. More rarely, as in Figs. 5 and 7, a gasp was seen when the inflation pressure was reduced. Occasionally the only gasp

seen was one occurring when respiration was resumed after a period of apnoea.

The inflations which gave rise to gasps were generally performed rapidly (Figs. 1, 5–7). To test whether the gasping response was due to the rate of

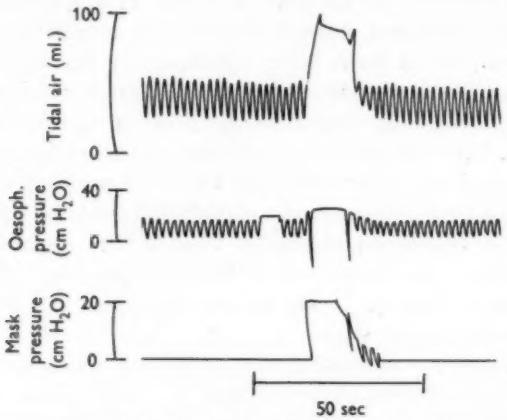

Fig. 5. Positive pressure inflation of baby No. 86, female, 3·3 kg, age 25 hr. Inflation produced a gasp followed by apnoea. When the pressure in the mask was allowed to fall, inadvertently, another gasp occurred.

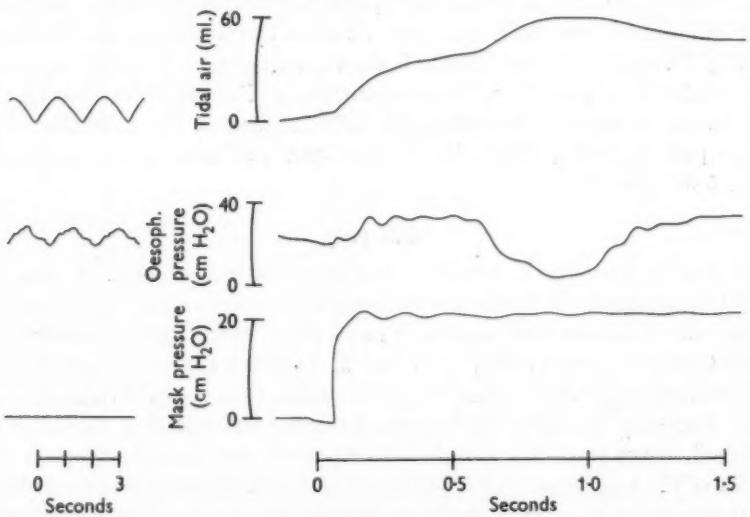

Fig. 6. Inflation, recorded at high speed, of baby No. 118, male, 4·7 kg, age 2 hr. The left side of the panel shows two respiratory cycles recorded three cycles before the inflation (different time scale). The time course of the inflation and the gasp are well shown.

inflation, two babies were inflated by gradual body decompression (e.g. -20 cm H_2O in 12 sec). With these slow inflations gasps still occurred readily (see Fig. 8).

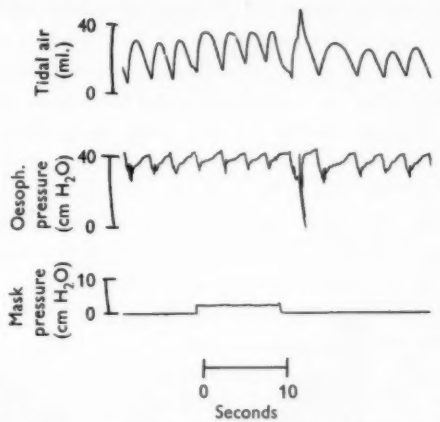

Fig. 7. Baby No. 114, female, 3·34 kg, age 1 hr. Note the occurrence of a gasp after the release of pressure (2·5 cm H_2O) in the mask. This is perhaps analogous to the second gasp seen in Fig. 5.

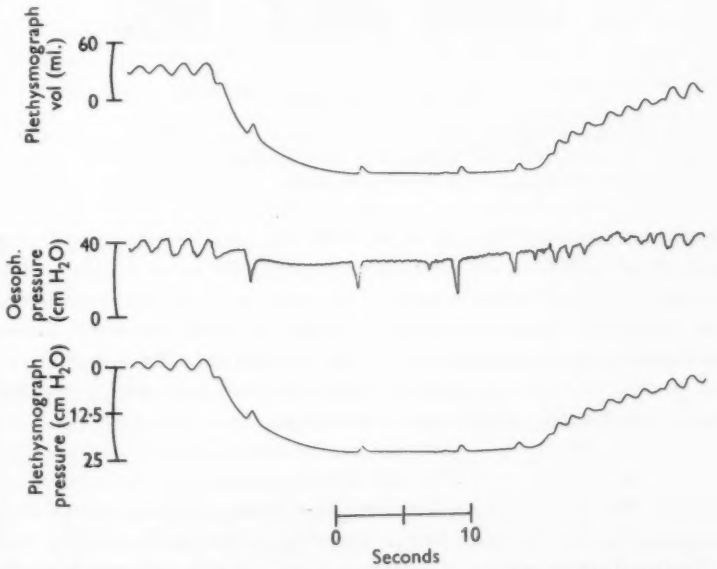

Fig. 8. Inflation of baby No. 139, male, 3·8 kg, age 5 hr. Note that this baby has been inflated by body decompression, and the inflation was undertaken deliberately slowly. Three gasps occurred during the inflation.

By far the most common gasp is that occurring initially. Figure 9 shows the incidence of initial gasps in babies who were rapidly inflated, both in relation to their age and to the pressure applied. The majority (80 %) of babies in our study under 24 hr of age gasped when inflated with

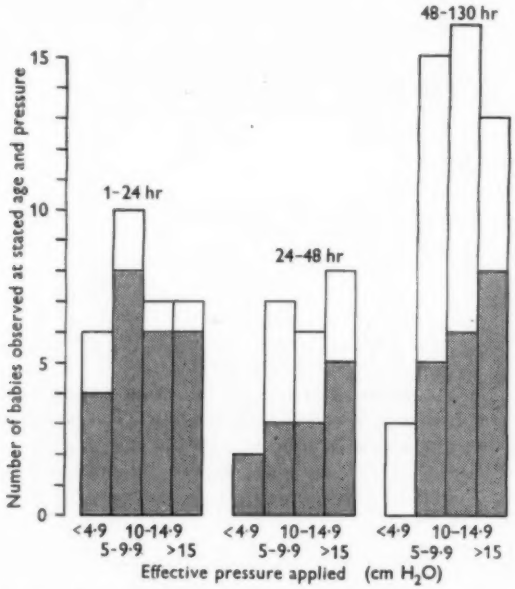

Fig. 9. Histogram showing the incidence of gasps occurring at given ages and pressures in the forty-three babies who were inflated rapidly. A significantly higher percentage of gasps occurred in the youngest babies. □, No gasp; ▤, gasp.

all pressures applied. Babies over 48 hr of age gasped less frequently (40 %). A χ^2 test between these two age groups gives a value of 13·5, $P < 0{\cdot}01$. Babies of all ages were more likely to gasp when subjected to higher pressures. A χ^2 test between gasps at pressures 0–4·9 and over 15 cm water is not significant. However, 89 % of the inflations done at the lowest pressures were on babies under 24 hr of age, and these babies tended to gasp with a minimum of stimulus.

DISCUSSION

Apnoea. We are unable to find that the Hering–Breuer reflex has been investigated in the new-born baby. Recently Dawes & Mott (1959) studied the Hering–Breuer reflex in the new-born rabbit and established the important point that it was present in this species, which is very immature at birth. The studies described here differ from most of the work on the

apnoeic reflex in that the babies were unanaesthetized. Ritzel (1950) has studied human adults during normal sleep as well as during anaesthesia with ether or barbiturates; he was unable to find a significant difference between the two groups in expiratory prolongation when the airway had been occluded at the height of a normal spontaneous inspiration. We were able to study, incompletely, one baby of 24 hr of age who had been anaesthetized with ether and had a tracheal intubation before an operation for imperforate anus. This baby was breathing rapidly and regularly at 54/min, and the body decompression showed both a gasp and brief apnoeic pauses (less than 4 sec), but as there was a leak around the baby's face, accurate quantitative data could not be obtained. Marshall & Widdicombe (1958) have reported that the Hering–Breuer reflex is weak, or sometimes absent, in man, and record a maximum apnoea of about 10 sec in one anaesthetized subject. The absolute time in the adult is a great deal shorter than that seen in many babies, and the ratio of the duration of apnoea to the normal respiratory cycle is about a quarter of the largest seen in a baby. Marshall & Widdicombe have expressed their results in relation to the transpulmonary pressure applied, but as we did not measure the oesophageal pressure in absolute terms we have preferred to examine our results more on the basis of volume than pressure. Bucher (1949), who investigated the reflex by the tracheal occlusion method (Head, 1889), also found a weak response in the human adult as compared with some other species (cat, rabbit, guinea-pig and rat). Christiansen & Haldane (1914) found much longer periods of apnoea in conscious human subjects, but it is uncertain whether such results are valid in conscious subjects who have some knowledge of physiology. It would seem, therefore, that the new-born baby responds to inflation by a longer inhibition of respiration, whether this is measured directly in seconds or whether it is expressed by the number of respiratory cycles which are lost. What is more surprising, perhaps, is to find that the duration of apnoea for a constant lung inflation is diminishing in babies over a period of 124 hr. Clearly there cannot be a linear change with age until adult life is reached, as an extrapolation from the slope shown in Fig. 4 would lead one to expect no Hering–Breuer reflex in the human subject after about 8 days of age.

The gasp. We have failed to find any report that a gasp reflex in response to inflation occurs in the adult human subject. Head (1889), in his work on the regulation of respiration, mentions three types of inspiratory activity which may occur in response to inflation. First is a gasp occurring at the end of an apnoea which Head refers to as 'an interrupting inspiration'. This he describes as a 'quick contraction, often double the strength of a normal inspiration'. Secondly, on page 17 of his paper, he says: 'I must here reiterate the warning of Hering and Breuer against violent inflation....

If the air is violently blown into the lungs, an inspiration always precedes the inhibitory pause. . . .It is in all probability due to stimulation of some sensory nerve in the body wall, for I have seen it in but lightly narcotized animals, even after division of the vagi.' By 'violent' inflation Head seems to have meant 'rapid' or 'large'. Yet some, at least, of our inflations cannot be so called, for Fig. 8 shows gasps occurring with a deliberately slow inflation, and a gasp has been recorded with a pressure as low as 2·2 cm of water. Figure 7 shows a gasp after the release of a mask pressure of only 2·5 cm of water. Finally, after 'freezing' the vagi with thermodes, and allowing them to recover 'conducting power' by 'moistening themwith a paint brush dipped in salt solution' Head noted that a stage of recovery appeared 'in which the inflation, instead of inhibiting the inspirations actually shortens the expiratory period. The strength of the inspiratory contractions is not increased, but their duration is prolonged, and owing to the incomplete expiratory elongation a tonic inspiratory contraction appears during the inflation'. Inspiration during this recovery phase he refers to as a 'paradoxical effect'.

Head's Paradoxical Reflex has been studied extensively by Larrabee & Knowlton (1946) and also by Widdicombe (1954*a*, *b*, *c*). Larrabee & Knowlton (1946) worked on cats whose body wall had been removed, and thus, when they found an increased phrenic discharge in response to inflation of the lungs, they could be confident that the afferent pathway was not in the body wall. In their later work, Knowlton & Larrabee (1946) identified vagal fibres whose endings were quick-adapting and responded to both inflation and deflation (unlike the Hering–Breuer sensory afferents) and which they thought to be the afferent pathway for Head's Paradoxical Reflex. Widdicombe, on the other hand, found that the receptors of Larrabee & Knowlton were probably cough receptors and tracheo-bronchial in origin. He obtained phrenic discharges in response to inflation of the deeper parts of the lung, but tracheo-bronchial inflation did not elicit this response. Thus in the cat the receptors for Head's Paradoxical Reflex are probably quick-adapting, are deep in the lung and respond only to moderately large inflations. The sensory fibres mediating this response all cease to conduct at 5° C and begin to be paralysed at 11° C, as judged by the cessation of reflex responses.

The gasp in response to inflation in the baby would appear to have two possible origins—either the body wall (Head, 1889) or the quick-adapting receptors of the lung (Larrabee & Knowlton, 1946; Widdicombe, 1954*d*). Evidence against the former is their occurrence with gradual inflations as opposed to the violent inflation described by Head (Fig. 8). If it is the latter, then it would be proper to refer to the gasp in the baby as a Head's Paradoxical Reflex. As we are unable to cut the vagi, or remove the body

wall of the new-born baby, it is difficult to design a definitive experiment to differentiate the possible receptor areas. In coming to the tentative conclusion that the gasps of babies in response to inflation are more likely to be mediated through the vagi than through body-wall afferents, it should be pointed out that the evidence, as it stands, is distinctly tenuous. The Paradoxical Response of the baby differs from that of the rabbit in that it is a gasp and not an increase in inspiratory tone. The Paradoxical Response of the cat does give a short burst of phrenic activity (as one would expect with a gasp) but the adequate stimulus in the baby seems to be different from that seen in the adult cat. Thus the baby may gasp with very small inflations, e.g. 2·2 cm water, and also an off-effect sometimes occurs, as in Figs. 5 and 7.

A further possibility is that the gasp of the baby is a response to cough receptors (?Larrabee & Knowlton, 1946) stimulated, for example, by abnormal air flow in the lungs. This seems unlikely, because although the normal baby can cough, there was never an active expiratory component in the responses seen. Gasps were elicited in a baby who had been anaesthetized and intubated under open ether, although cough receptors become less sensitive under ether (Whitteridge & Bülbring, 1944; Widdicombe, 1954*b*).

Whatever the sensory pathway of the gasp may be, it is of interest that the baby responds in this fashion. This response was significantly more frequent in the youngest babies and any stimulus which causes the baby to gasp must be taken into account when considering the mechanism of the first breath and lung expansion. Intrathoracic-pressure swings before the first breath have been observed by Karlberg (1957), and Bosma, Lind & Gentz (1959) have some radiographic evidence suggesting that babies make 'frog swallowing' movements before the onset of respiration. If there are changes of pressure, as suggested by these authors, they could stimulate the tracheo-bronchial receptors.

The effect of age, during the first 5 days of life, on both the Hering–Breuer reflex and on the gasp reflex is of some interest. Previous work by Miller & Smull (1955) and by Cross, Tizard & Trythall (1958) has suggested that the medullary respiratory centre of the new-born infant becomes more reactive to sensory bombardment of chemoreceptor origin during the first few days of life. Our present data, however, give no evidence as to whether a particular stimulus, such as a given volume of inflation, causes the same number of afferent impulses at different ages, or not. At a time like this, shortly after the baby's lungs had been expanded, it is possible that the compliance of the lungs might be altering, and that an apparent change in reflex behaviour might have much more to do with the physical properties of the lung than with any change of peripheral sensitivity or central

reactivity. For this reason we have examined the total compliance of the babies in relation to the factors already discussed, and we have been unable to find any consistent relationship.

SUMMARY

1. The respiratory response of the new-born infant to lung inflation has been examined. A marked apnoeic response occurred.

2. Frequently there is a gasp, which may be an example of the Head's Paradoxical Reflex, or may be mediated through body-wall afferents.

3. The duration of the apnoeic response and the incidence of the gasping response diminish with age.

It is a pleasure to thank Dr J. H. Comroe, Jr., for advice and encouragement in this work; Drs E. B. Shaw and E. Page for allowing us to examine their patients; and Mrs McAndrews and Mrs Francis and their staff for their day-to-day collaboration. This work was supported in part by Research Grant H. 4029 from the National Heart Institute of the USPHS to the University of California Medical Center, San Francisco. During this work K.W.C. and M.K. held United States Public Health Service Post-Doctoral Fellowships; W.H.T. held a Giannini Foundation Research Fellowship in Pediatrics.

REFERENCES

Bosma, J. F., Lind, J. & Gentz, N. (1959). Motions of the pharynx associated with initial aeration of the lungs of the newborn infant. *Acta paed. scand.* **48**, Suppl. 117–122.

Bucher, K. (1949). Vergleichende Charakterisierung der Lugenatmung einiger Säuger. *Helv. physiol. acta*, **7**, 470–475.

Christiansen, J. & Haldane, J. S. (1914). The influence of distention of the lungs on human respiration. *J. Physiol.* **48**, 272–277.

Cross, K. W. (1949). The respiratory rate and ventilation of the newborn baby. *J. Physiol.* **109**, 459–474.

Cross, K. W., Dawes, G. S. & Mott, J. C. (1959). Anoxia, oxygen consumption and cardiac output in new-born lambs and adult sheep. *J. Physiol.* **146**, 316–343.

Cross, K. W., Klaus, M., Tooley, W. H. & Weisser, K. (1959*a*). The response of the newborn infant to inflation of the lungs. *A.M.A. J. Dis. Child.* **98**, 463.

Cross, K. W., Klaus, M., Tooley, W. H. & Weisser, K. (1959*b*). The gasp of the new-born infant in response to lung inflation. *J. Physiol.* **149**, 53–54*P*.

Cross, K. W., Tizard, J. P. M. & Trythall, D. A. H. (1958). The gaseous metabolism of the newborn infant breathing 15% O_2. *Acta paediat., Stockh.*, **47**, 217–237.

Dawes, G. S. & Mott, J. C. (1959). Reflex respiratory activity in the new-born rabbit. *J. Physiol.* **145**, 85–97.

Dornhorst, A. C. & Leathart, G. L. (1952). A method of assessing the mechanical properties of the lungs and air passages. *Lancet*, **263**, 109–111.

Head, H. (1889). On the regulation of respiration. *J. Physiol.* **10**, 1–70.

Karlberg, P. (1957). In *Physiology of Prematurity*. Trans. II Conf. Josiah Macy, Jr. Foundation, ed. Lanman, J. T. New York: Josiah Macy, Jr. Foundation.

Knowlton, G. C. & Larrabee, M. G. (1946). A unitary analysis of pulmonary volume receptors. *Amer. J. Physiol.* **147**, 100–114.

Larrabee, M. G. & Knowlton, G. C. (1946). Excitation and inhibition of phrenic motoneurones by inflation of the lungs. *Amer. J. Physiol.* **147**, 90–99.

Lee, G. de J. & DuBois, A. B. (1955). Pulmonary capillary blood flow in man. *J. clin. Invest.* **34**, 1380–1390.

Marshall, R. & Widdicombe, J. G. (1958). The weakness of the Hering–Breuer reflexes in man. *J. Physiol.* **140**, 36*P*.

MILLER, H. C. & SMULL, N. W. (1955). Further studies on the effects of hypoxia on the respiration of newborn infants. *Pediatrics*, **16**, 93.

RITZEL, G. (1950). Über die vagale Atmungssteuerung des Menschen. *Schweiz. Z. Tuberk.* **7**, 193–204.

WHITTERIDGE, D. & BÜLBRING, E. (1944). Changes in activity of pulmonary receptors in anaesthesia and their influence on respiratory behaviour. *J. Pharmacol.* **81**, 340–359.

WIDDICOMBE, J. G. (1954*a*). Respiratory reflexes from the trachea and bronchi of the cat. *J. Physiol.* **123**, 55–70.

WIDDICOMBE, J. G. (1954*b*). Receptors in the trachea and bronchi of the cat. *J. Physiol.* **123**, 71–104.

WIDDICOMBE, J. G. (1954*c*). Respiratory reflexes excited by inflation of the lungs. *J. Physiol.* **123**, 105–115.

WIDDICOMBE, J. G. (1954*d*). The site of pulmonary stretch receptors in the cat. *J. Physiol.* **125**, 336–351.

J. Physiol. (1960), **151**, *pp.* 566–577
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THE EFFECTS OF CALCIUM AND MAGNESIUM ON MAMMALIAN MUSCLE FIBRES

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During an investigation into the neuromuscular block produced by magnesium in the rat diaphragm, it was found that although calcium reversed the effect of magnesium on neuromuscular transmission, it failed to restore the twitch height to its original level (Paul, 1957). A considerable number of workers have found that calcium and magnesium depress the excitability of amphibian muscle (Engbaek, 1948; Jenerick & Gerard, 1953; del Castillo & Engbaek, 1954), but little work appears to have been done on mammalian muscle, most of the earlier work being concerned with the anaesthetic action of magnesium in whole animals. However, Nothman (1921) reported that high blood calcium concentrations reduced the excitability of human skeletal muscle, and Maaske & Gibson (1939) found that magnesium reduced the twitch height of directly stimulated and denervated dog muscle, owing, they concluded, to an increase in threshold.

The effects of calcium and magnesium on an isolated mammalian muscle, namely, the rat phrenic-nerve–diaphragm preparation, are described in the present paper. An account of part of this work has been previously submitted to the University of London in part fulfilment of the requirements for the degree of Doctor of Philosophy.

METHODS

A modified version of the technique introduced by Bülbring (1946) and subsequently developed by McDowall, Miechowski & Shafei (1949) and Hadju & McDowall (1949) was employed. Diaphragm muscles from albino rats were dissected complete, together with both phrenic nerves, and placed in oxygenated Krebs's solution, in which all further handling was carried out. A rectangular segment was cut out, as this preserved as many fibres intact as possible, damage being confined to the two parallel cuts made along the line of the fibres. The entry of the phrenic nerve was included in this segment. The preparation was attached to a standard plastic diaphragm electrode (Bell, 1953) by the central tendon, and the costal margin was attached to the recording system. The nerve was laid over a pair of platinum electrodes, and the plastic holder was suitably wired to allow independent direct stimulation of the muscle, again through platinum electrodes.

The preparation was immersed in a 50 ml. organ bath filled with Krebs's solution of composition (mM): Na^+ 145, K^+ 5·9, Ca^{2+} 2·6, Mg^{2+} 1·2, Cl^- 128·9, $H_2PO_4^-$ 1·2, SO_4^{2-} 1·2, HCO_3^- 25·0, glucose 5·5 (Krebs & Henseleit, 1932). To increase the concentration of an ion during an experiment, suitable amounts of a molar solution of its chloride were added to the bath. The resulting increase in volume was kept to a maximum of 1·5 ml. or less throughout the experiment. Aeration was carried out with a 95 % O_2 + 5 % CO_2 gas mixture, bubbled through a plastic tube plugged with fluted glass. The gas bubbles were made to impinge on the tissue to ensure adequate oxygenation (Creese, Scholes & Whalen, 1958), and they also served to mix the contents of the bath. The pH of the aerated solution was 7·4. The bath was warmed by a heated water jacket and kept at 37–38° C. The nerve was also immersed and was stimulated below the surface of the bath fluid.

Stimulation was performed by means of square pulses of a constant voltage, which was set to give a maximal twitch response for each individual muscle in normal Krebs's solution. Impulse duration was 0·2 msec for nerve and 2 msec for direct stimulation. Stimulation frequency was 15–16/min, at which rate a preparation normally continued to respond for at least 8 hr.

In most cases contractions were recorded with a simple isotonic lever. Dimensions were: muscle attachment to fulcrum 2 cm; fulcrum to writing point 18 cm; total weight of unloaded lever 1·25 g.

Estimations of potassium and sodium were made with an EEL flame photometer. Tissue samples were blotted dry, weighed, and dried at 120° C for 15–20 hr. After cooling in a desiccator the samples were reweighed and dissolved in 50 % (v/v) nitric acid at 100° C on a water-bath. The resulting solution was diluted to 50 ml. for analysis. The solutions used for the soaking experiments were prepared with the concentrations already adjusted. In these experiments increases in concentration of ions were not accompanied by dilution of the solution.

RESULTS

Effects of calcium and magnesium on the twitch height

Additions of either calcium or magnesium to the bath depressed the twitch height of an active preparation. Figure 1 shows the effect of increasing the bath concentration of calcium or magnesium by 4 mM. The preparation had been curarized to block neuromuscular transmission with a bath concentration of 2×10^{-5} D-tubocurarine, and was stimulated directly. This was necessary to show the effect of magnesium on the muscle fibres as distinct from its blocking action on neuromuscular transmission (Liley, 1956; Paul, 1957).

Preparations readily recover from a depression induced by calcium or magnesium when washed with normal Krebs's solution. Both depression and recovery take place more rapidly with calcium than with magnesium. As the hydrated calcium ion is rather smaller than the hydrated magnesium ion, this might be attributable to a difference in diffusion rate into the tissue. The relative ionic radii, based on potassium as unity, have been given by Conway (1945) as Ca^{2+} 2·51, and Mg^{2+} 2·64.

Progressive increases in the calcium or magnesium concentrations produce corresponding decreases in the twitch height, until the total depression is of the order of 30 % with calcium and 50 % with magnesium. The

majority of preparations reach maximum depression with 20 mM excess calcium or magnesium.

Most of these experiments were performed on curarized preparations, but the calcium depression could be obtained equally well on indirectly stimulated preparations. Several reports have been published of neuromuscular blocks produced by excess calcium in amphibian nerve–muscle preparations (Locke, 1894; Mines, 1908; Coppée, 1946; Hunt & Kuffler, 1950), but this could not be repeated for rat muscle. Excess calcium never abolished the response completely.

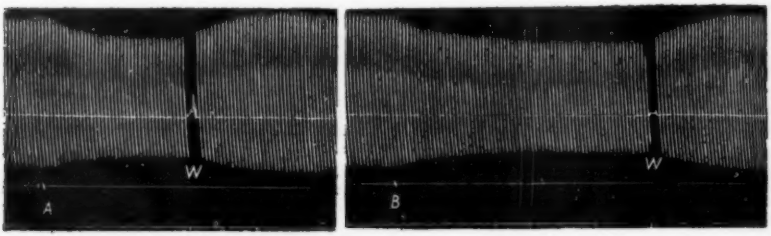

Fig. 1. Depression due to increase of Ca^{2+} and Mg^{2+}. The preparation had been curarized. *A*, Ca^{2+} concentration increased by 4 mM. At *W* preparation washed with normal Krebs's solution. *B*, Mg^{2+} concentration increased by 4 mM. At *W* preparation washed with normal Krebs's solution. Time trace shows 1 min intervals.

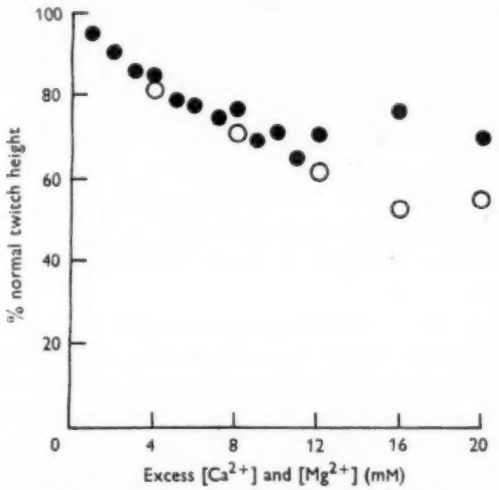

Fig. 2. Progressive depression of twitch height with increase of Ca^{2+} or Mg^{2+} until maximum depression was reached. Each point represents the mean value of at least four observations. Ca^{2+} and Mg^{2+} concentrations are plotted as mM excess over the normal concentration. Twitch height is plotted as a percentage of the initial twitch height. Ca^{2+} ●; Mg^{2+} ○.

The effect of potassium on depressions due to calcium and magnesium

The effect of calcium on a preparation is different when the experiment is carried out in Krebs's solution with an increased potassium concentration, but the effect of excess magnesium is not altered. After the potassium concentration has been raised an increase in calcium concentration produces first a decrease in twitch height (as in normal Krebs's solution), followed by a slow increase in twitch tension which may take 10 min or more to reach its maximum. Figure 3 shows the response of a preparation to four successive increases of calcium concentration, each of 4 mM, after the potassium concentration had previously been increased by 4 mM.

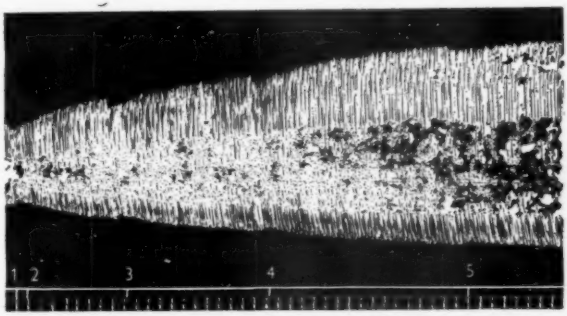

Fig. 3. The effect of successive 4 mM increases of Ca^{2+} concentration after the potassium concentration had been increased by 4 mM. At signals 2, 3, 4, and 5 the calcium concentration was increased by 4 mM. The signal is displaced to the right by the distance between 1 and 2. The twitch height had been depressed 44 % by the potassium and was increased finally to 103 % normal by the addition of Ca^{2+}. Time trace: 1 min intervals.

By using two preparations from the same animal the twitch heights at various levels of calcium and magnesium concentration both before and after an increase in potassium concentrations can be obtained. Figures 4 and 5 show the twitch height, as a percentage of the original twitch height, plotted against the calcium and magnesium concentrations, respectively, both before and after the potassium concentration was raised by 4 mM. The magnesium depression was similar in both cases, but the calcium depression was reversed with increased potassium.

Potassium has been shown to have a twofold effect on the rat diaphragm, depending on the concentration; at low concentrations the twitch height is increased, and at higher concentrations the twitch height is depressed (Hadju, Knox & McDowall, 1950). The absolute potassium concentration at which augmentation of the twitch is superseded by depression varies considerably from preparation to preparation, so that in the present

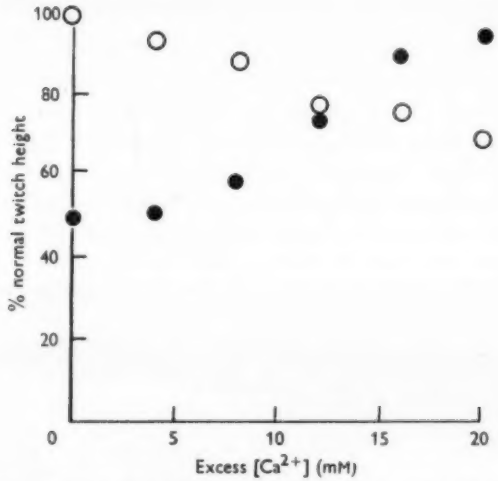

Fig. 4. Effect of increasing Ca^{2+} concentration before and after the potassium concentration had been increased by 4 mM. ○, normal Krebs's solution; ●, plus 4 mM potassium.

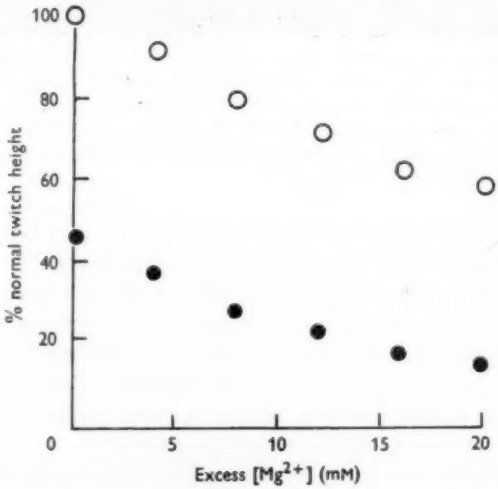

Fig. 5. Effect of increasing Mg^{2+} concentration before and after the potassium concentration had been increased by 4 mM. ○, normal Krebs's solution; ●, plus 4 mM potassium. This preparation was from the same animal as that in Fig. 4.

experiments increasing the potassium concentration by 4 mM usually resulted in depression but sometimes in a maintained increase in twitch height. In the latter case increasing the calcium concentration produced its typical response of depression followed by augmentation. The final increase in twitch height exceeded that produced by the potassium alone.

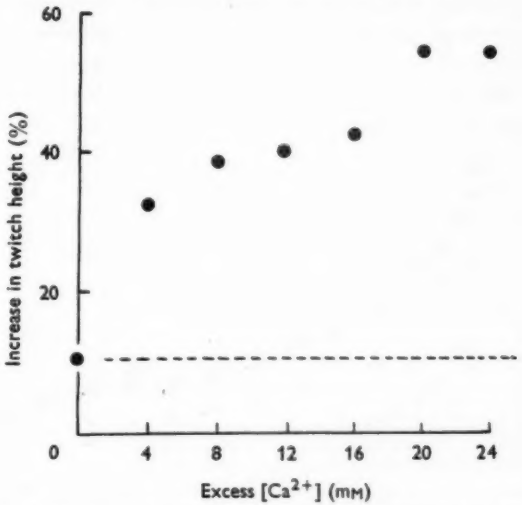

Fig. 6. Effect of increase of Ca^{2+} concentration after the potassium concentration had been increased by 4 mM, but had not produced any depression. Broken line indicates level to which twitch height was increased by the potassium. Ordinate gives percentage increase over initial twitch height.

Effects of calcium on post-tetanic potentiation

As post-tetanic potentiation has been ascribed to the accumulation of potassium ions in the extracellular muscle spaces (Brown & von Euler, 1938), experiments were carried out to see whether calcium affected this action. A muscle was tetanized for 10 sec at a frequency of 5 impulses/sec. A low frequency was used to avoid fatiguing the muscle unduly, as the procedure was repeated several times. The amount of potentiation after the tetanus was measured during the time taken for the twitch height to return to normal. Figure 7 shows the relationship between potentiation and time after a tetanus, for various concentrations of calcium. The higher the calcium concentration, the longer the potentiation lasts, and the greater is the degree of potentiation. An increase in magnesium concentration of 4 mM did not produce a detectable alteration of post-tetanic potentiation. These results clearly resemble the action of potassium on the calcium depression described above.

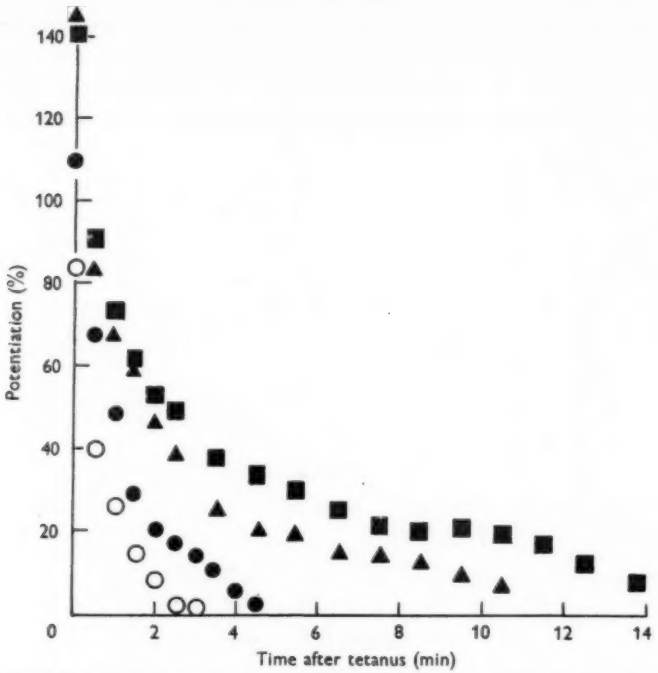

Fig. 7. Effect of Ca^{2+} on post-tetanic potentiation. Potentiation is plotted as percentage increase in twitch height over the initial level. ○, normal Krebs's solution; ●, plus 4 mM-Ca^{2+}; ▲, plus 8 mM-Ca^{2+}; ■, plus 12 mM-Ca^{2+}.

Effect of calcium on the potassium and sodium content of muscle

As the potassium ion is known to affect the twitch height of the rat diaphragm (Hadju *et al.* 1950) experiments were carried out to see whether calcium had any effect on the tissue content of this ion. Potassium movements often compensate for movements of sodium, and that ion was also estimated at the same time.

Diaphragms were divided into four sections, and one section from each muscle was soaked in one of the following solutions: normal Krebs's solution, and Krebs's solution containing 2, 3, or 5 times normal calcium concentration. The muscles were soaked for 1·5 hr at 37·5° C, and then analysed for potassium and sodium content. The results are given in Table 1. No statistically significant difference was found between muscles soaked in normal Krebs's solution and those soaked in high-calcium solutions.

In a further series of experiments segments from each diaphragm were

soaked for 1 hr in normal Krebs's solution, in solutions with twice normal Ca^{2+}, with twice normal K^+, and in a solution with twice normal Ca^{2+} as well as twice normal K^+. The results are shown in Table 2. Doubling the potassium concentration produced a fall in tissue potassium content of about 7–8 m-equiv/kg wet tissue. The sodium concentration increased by a similar amount. Both the decreased tissue potassium and the increased tissue sodium were significantly different from the normal value (for K^+, $P = 0{\cdot}02$–$0{\cdot}01$; for Na^+, $P = 0{\cdot}05$–$0{\cdot}02$). Assuming a constant value of 0·28 for extracellular space (see Creese *et al.* 1958) and the values for water content of the tissues given in the table, the fall in tissue potassium

TABLE 1. Tissue content of sodium and potassium with different concentrations of Ca^{2+} in the soaking solution. Results are expressed as mean ± S.E. of mean, with number of observations in brackets. In no case is the value for increased Ca^{2+} significantly different from the control. The water content of the tissues is also given. Tissues were soaked for 1·5 hr

Soaking solution	Tissue concentrations (m-equiv/kg wet tissue) Na	K	Water content (% wet weight)
Normal Krebs's solution (control)	69·2 ± 3·5 (8)	52·0 ± 1·9 (8)	76·4 ± 0·03 (8)
$2 \times Ca^{2+}$	65·4 ± 2·7 (9)	54·4 ± 1·7 (9)	76·0 ± 0·25 (9)
$3 \times Ca^{2+}$	71·8 ± 3·9 (7)	50·8 ± 2·6 (7)	76·4 ± 0·17 (7)
$5 \times Ca^{2+}$	69·6 ± 6·0 (5)	55·5 ± 3·1 (5)	76·6 ± 0·20 (5)

TABLE 2. Effects of increasing Ca^{2+} and K^+ concentrations on the tissue content of potassium and sodium. Results as in Table 1. The tissues were soaked for 1 hr

Soaking solution	Tissue concentrations (m-equiv/kg wet tissue) Na	K	Water content (% wet weight)
Normal Krebs's solution (control)	65·4 ± 1·7 (17)	71·2 ± 1·4 (17)	77·1 ± 0·1 (17)
$2 \times Ca^{2+}$	68·0 ± 3·7 (18)	71·8 ± 1·0 (18)	76·7 ± 0·1 (18)
$2 \times K^+$	73·4 ± 2·9 (20)	64·1 ± 2·3 (20)	77·6 ± 0·1 (20)
$2 \times Ca^{2+}$ and $2 \times K^+$	73·1 ± 2·9 (15)	63·7 ± 3·0 (15)	78·0 ± 0·1 (15)

can be interpreted as a fall in intracellular potassium concentration of about 13–14 m-equiv/l. fibre water. The intracellular sodium rises by a similar amount. This effect of twice normal potassium was not altered by doubling the calcium concentration.

A few experiments with a mechano-electric transducer confirmed that the effects of the solutions used for the soaking experiments resembled the results recorded with the isotonic lever.

DISCUSSION

Both calcium and magnesium have a considerable depressive effect on rat-diaphragm muscle fibres. As the depression appears within seconds of adding the ions to the bath the action almost certainly takes place at the

fibre membrane. Numerous observations have been made of a similar action of these ions on amphibian muscle, and it has been demonstrated that high calcium and magnesium concentrations decrease the excitability of the fibre membrane by raising the threshold to stimulation (Chao, 1935; Ashkenaz, 1938; Carleton, Blair & Latchford, 1938; Jenerick & Gerard, 1953; del Castillo & Engbaek, 1954). Weidmann (1955) has observed a similar effect on mammalian Purkinje fibres, finding that a greater degree of depolarization is required for excitation when the calcium or magnesium concentration is raised. A similar effect of calcium on nerve is well known (literature reviewed by Brink, 1954).

The decrease in excitability with increased calcium concentration is not so marked in the rat diaphragm as in frog muscle. Several observations of neuromuscular blocks with high calcium concentrations have been made (Locke, 1894; Mines, 1908; Coppée, 1946), but the experiments of Hunt & Kuffler (1950) using the frog sartorius preparation have demonstrated that the end-plate potential could still be obtained when the muscle, under the influence of high calcium, had ceased to respond to stimulation. The apparent block was due to a big decrease in excitability of the fibre membrane. Engbaek (1948) was able to show that magnesium in high concentrations also prevented the spread of excitation from the end-plate in frog muscle. In the rat diaphragm complete loss of excitability with high calcium or magnesium was never seen, the maximum depression ever recorded being 65% with 16 mM excess magnesium. Maaske & Gibson (1939) were also unable to depress completely the response of denervated dog muscle with magnesium.

This difference in susceptibility between frog and rat muscle may be due to the smaller factor by which the calcium concentration was raised in these present experiments. Coppée (1946) used 16 times Ringer's concentration of calcium to produce a block, and this is equivalent to a concentration of 27·8 mM. A comparable increase, i.e. 16 times Krebs's concentration, would be 39·6 mM calcium, but the calcium concentration of Krebs's solution cannot be raised much above 30 mM without the salt being precipitated, so that the required concentration cannot be reached. The observed difference between the effects of equal amounts of calcium and magnesium at high concentrations (Fig. 2) may be accounted for by a similar argument. As the normal calcium concentration is more than twice that of magnesium, equal amounts of these ions do not increase the respective concentrations by the same factor, that for magnesium being twice that for calcium.

The effect of an increase in the potassium concentration on the response to calcium resembles the observations of Niedergerke (1956), that calcium enhances the tension developed by an isolated frog's heart when the fibres

have been partly depolarized by potassium. In the rat diaphragm also calcium enhances the tension developed after the fibres have been subjected to an increased potassium concentration. From the usual Nernst equation the theoretical decrease in membrane potential due to a doubling of external potassium concentration would be about 18 mV. The accompanying fall in the intracellular potassium concentration by 13 m-equiv/l. is not what would be expected to occur on *a priori* grounds. The membrane potential might be expected to decrease, and the intracellular potassium concentration to show a slight increase. A possible explanation of the observed fall in concentration could be that the lowering of the membrane potential by potassium increases the leakiness of the membrane to sodium, and loss of potassium would then compensate for the increased entry of sodium.

Calcium itself does not produce any alteration in the potassium and sodium contents, and its augmenting action with increased potassium concentrations is not duplicated by magnesium. This leads to the conclusion that a specific action of the calcium ion is involved. Apparently potassium sensitizes the muscle to calcium, revealing an action that is masked under normal circumstances. The experiments on post-tetanic potentiation suggest that a similar effect is obtained when the potassium concentrations inside and outside the cell are varied in a more physiological way.

Antagonisms between potassium and calcium in a variety of tissues have been described (Ringer, 1883; Mines, 1908, 1912; Libet & Gerard, 1939). However, in the present case a simple antagonism between calcium and potassium affecting the twitch height is not operating. Calcium does not simply reverse the effect of potassium.

Micro-injection of calcium into muscle fibres causes the fibrils to shorten in the region of the injection (Heilbrunn & Wiercinski, 1947; Niedergerke, 1955). The activity of squid giant nerve fibres has been shown to be accompanied by an influx of calcium into the axoplasm (Hodgkin & Keynes, 1957), and calcium is also released to the exterior as a result of muscular activity (Heilbrunn, 1956). The recent experiments of Bianchi & Shanes (1959) on the entry of labelled calcium into active muscle have enabled the authors to support the prediction that entry of calcium into muscle fibres underlies the development of a contractile response. These reports strongly suggest that activity is accompanied by movements of calcium, and that contraction may depend on calcium entering the muscle fibre. If calcium is concerned with contraction in this way the effect of potassium might be to increase the movement of calcium into the cell during activity.

SUMMARY

1. The effects of calcium and magnesium ions on mammalian muscle fibres have been studied with the rat phrenic-nerve–diaphragm preparation.

2. Both calcium and magnesium depress the twitch height of a contracting diaphragm by affecting the muscle fibres directly. The depression reaches a maximum of 30 % with calcium and 50 % with magnesium when the concentration of either is increased by 16–20 mM.

3. With twice normal extracellular [K], calcium produces a rise in twitch height while magnesium still depresses it. A fall in tissue potassium content of about 7 m-equiv/kg wet weight also occurs in high [K], together with a corresponding rise in tissue sodium content.

4. Calcium has no effect on potassium and sodium contents.

5. Post-tetanic potentiation is increased both in magnitude and duration by increased calcium concentration. This again suggests that with a small increase in the extracellular potassium concentration the muscle fibres are able to develop more tension than normal in the presence of high calcium concentrations.

I am grateful to Mr S. V. Hill for the design and construction of a mechano-electric transducer, and to Drs W. T. W. Potts and R. W. Murray for much helpful discussion and for reading the manuscript.

REFERENCES

ASHKENAZ, E. (1938). Magnesium narcosis in muscles. *J. cell. comp. Physiol.* **11**, 163–174.

BELL, G. H. (1953). *Experimental Physiology*, 5th ed., p. 37. Glasgow: Smith and Son.

BIANCHI, C. P. & SHANES, A. M. (1959). Calcium influx in skeletal muscle at rest, during activity and during potassium contracture. *J. gen. Physiol.* **42**, 803–815.

BRINK, F. (1954). The role of calcium ions in neural processes. *Pharmacol. Rev.* **6**, 243–298.

BROWN, G. L. & VON EULER, U. S. (1938). The after effects of a tetanus on a mammalian muscle. *J. Physiol.* **93**, 39–61.

BÜLBRING, E. (1946). Observations on the isolated phrenic nerve diaphragm preparation of the rat. *Brit. J. Pharmacol.* **1**, 38–61.

CARLETON, B. H., BLAIR, H. A. & LATCHFORD, W. B. (1938). The effects of the chlorides of K, Ca, and Na on the excitability of muscle. *J. cell. comp. Physiol.* **12**, 223–236.

CHAO, I. (1935). Action of electrolytes on the electrical stimulation of skeletal muscle. *J. cell. comp. Physiol.* **6**, 1–19.

CONWAY, E. J. (1945). The physiological significance of inorganic levels in the internal medium of animals. *Biol. Rev.* **20**, 56–72.

COPPÉE, G. (1946). Le rôle des ions calcium dans la transmission neuromusculaire. *Arch. int. Physiol.* **6**, 1–19.

CREESE, R., SCHOLES, N. W. & WHALEN, W. J. (1958). Resting potentials of diaphragm muscle after prolonged anoxia. *J. Physiol.* **140**, 301–317.

DEL CASTILLO, J. & ENGBAEK, L. (1954). Nature of the neuromuscular block produced by magnesium. *J. Physiol.* **124**, 370–384.

ENGBAEK, L. (1948). Investigations on the course of and localisation of magnesium anesthesia: a comparison with ether anesthesia. *Acta pharm. tox., Kbh.*, **4**, Suppl. 1.

HAJDU, S., KNOX, J. A. C. & MCDOWALL, R. J. S. (1950). Potassium and neuromuscular transmission. *J. Physiol.* **111**, 382–387.

HAJDU, S. & MCDOWALL, R. J. S. (1949). The dependence of neuromuscular transmission on glucose. *J. Physiol.* **108**, 502–507.

HEILBRUNN, L. V. (1956). *The Dynamics of Living Protoplasm*, pp. 109–110. New York: Academic Press Inc.

HEILBRUNN, L. V. & WIERCINSKI, F. J. (1947). The action of various cations on muscle protoplasm. *J. cell. comp. Physiol.* **29**, 15–32.

HODGKIN, A. L. & KEYNES, R. D. (1957). Movements of labelled calcium in squid giant axons. *J. Physiol.* **138**, 253–281.

HUNT, C. C. & KUFFLER, S. W. (1950). Pharmacology of the neuromuscular junction. *Pharm. Rev.* **98**, 96–120.

JENERICK, H. & GERARD, R. W. (1953). Membrane potential and threshold of single muscle fibres. *J. cell. comp. Physiol.* **42**, 79–102.

KREBS, H. A. & HENSELEIT, K. (1932). Untersuchungen über die Harnstoffbildung im Tierkorper. *Hoppe-Seyl. Z.* **210**, 33–66.

LIBET, B. & GERARD, R. W. (1939). Control of the potential rhythm of the isolated frog brain. *J. Neurophysiol.* **2**, 153–169.

LILEY, A. W. (1956). Quantal components of the mammalian end-plate potential. *J. Physiol.* **133**, 571–587.

LOCKE, F. S. (1894). Notiz über den Einfluss physiologischer Kochsolzlösung auf die elektrische Erregbarkeit von Muskel und Nerv. *Zbl. Physiol.* **8**, 166–167.

MAASKE, C. & GIBSON, B. (1939). The effects of magnesium upon denervated mammalian muscle. *Amer. J. Physiol.* **127**, 486–491.

MCDOWALL, R. J. S., MIECHOWSKI, W. & SHAFEI, A. Z. (1949). The effects of glucose on the action of the rat diaphragm. *J. Physiol.* **108**, 24–32.

MINES, G. R. (1908). On the spontaneous movements of amphibian skeletal muscle in saline solutions, with observations on the influence of potassium and calcium chloride on muscular excitability. *J. Physiol.* **37**, 408–444.

MINES, G. R. (1912). On the relations to electrolytes of the hearts of different species of animals. I. Elasmobranchs and Pecten. *J. Physiol.* **43**, 467–506.

NIEDERGERKE, R. (1955). Local muscular shortening by intracellularly applied calcium. *J. Physiol.* **128**, 12–13*P*.

NIEDERGERKE, R. (1956). The potassium chloride contracture of the heart and its modification by calcium. *J. Physiol.* **134**, 584–599.

NOTHAM, M. (1921). Die galvanische Erregbarkeit des menschlischen Skelettmuskels nach intravenoser Zufuhr hochkonzentrierter Calciumlosungen. *Arch. exp. Path. Pharmak.* **91**, 312–316.

PAUL, D. H. (1957). The antagonism between magnesium and calcium at the neuromuscular junctions of the rat diaphragm. *J. Physiol.* **136**, 9–10*P*.

RINGER, S. (1883). A further contribution regarding the influence of the constituents of the blood on the contraction of the heart. *J. Physiol.* **4**, 29–42.

WEIDMANN, S. (1955). Effects of calcium ions and local anaesthetics on the electrical properties of Purkinje fibres. *J. Physiol.* **129**, 568–582.

J. Physiol. (1960), **151**, *pp.* 578–590
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HORMONAL CONTROL OF PREGNANCY BY THE OVARY AND PLACENTA IN THE RABBIT

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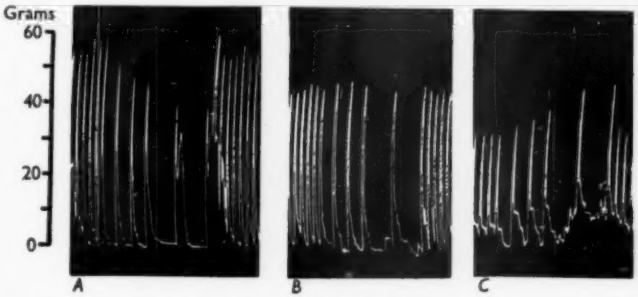
It has been shown (Bengtsson, 1957; Schofield, 1957) that in the rabbit progesterone influence is high during pregnancy, and as a consequence the myometrium is quiescent (Csapo, 1956*a*) during the development of the foetus. Immediately before parturition, however, the progesterone influence diminishes and the myometrium becomes fully reactive, allowing parturition to take place. Oestrogen levels apparently rise during pregnancy (Schofield, 1957), and hence when the progesterone 'block' (Csapo, 1956*a*) is removed at the end of pregnancy the contractile system of the myometrium is fully developed (Csapo & Corner, 1953). Progesterone, therefore, not only protects the foetus during pregnancy from excessive myometrial activity, but also influences the time of onset of parturition; this indicates that in the rabbit progesterone is the principal factor which determines the course of pregnancy and parturition.

In most species progesterone is known to be elaborated in relatively large amounts during pregnancy, but the source of the hormone is not always the same. In the human female, horse, cat and guinea-pig, for example, after a certain stage of gestation the ovaries can be excised without altering the course of pregnancy. It appears, therefore, that in these species the hormonal functions of the ovary have been taken over by some extragonadal source; the available evidence suggests that this source is the placenta and that by this stage of pregnancy the placenta is able to produce progesterone in amounts sufficient to maintain pregnancy. In other species, for example the rabbit, the ovary is indispensable at all stages of pregnancy and its removal results, within a day or two, in total abortion. In this animal the placenta is not able independently to maintain the required hormonal levels and hence, after ovariectomy, pregnancy is terminated.

This evidence points to three possible modes of placental function in the rabbit: (1) the placenta secretes no progesterone in normal pregnancy; (2) the placenta contributes only part of the total amount of progesterone necessary for the maintenance of pregnancy, and this may or may not be

dependent upon intact ovaries; or (3) all the progesterone is secreted by the placenta but its production is dependent upon intact ovaries and is not maintained after ovariectomy. The following experiments were designed to elucidate which of these mechanisms operates in the control of pregnancy in the rabbit.

In a general evaluation of the influence of ovarian hormones on myometrial function in the intact animal (Schofield, 1954, 1955) it was found that the functional response which most accurately reflects hormonal dominance is the staircase effect. A positive staircase effect (positive correlation between frequency of stimulation and tension developed) indicates oestrogen dominance, a negative staircase effect (negative correlation



Text-fig. 1. Tracing to show the three types of staircase effect. *A* is a positive staircase effect indicating oestrogen domination. *B* is a transient staircase effect showing that neither hormone is dominant. *C* is a negative staircase effect indicating progesterone domination.

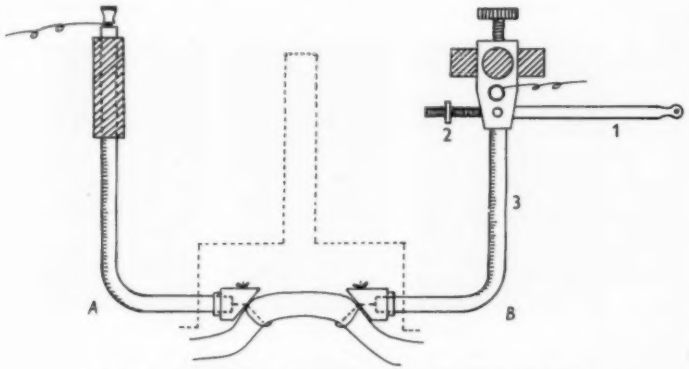
between frequency of stimulation and tension developed) indicates progesterone dominance, and a transient staircase effect (no slope) indicates that neither hormone is dominant (Text-fig. 1). This response has already been employed to indicate hormone dominance during the course of pregnancy in the rabbit (Bengtsson, 1957; Schofield, 1957), and it appears to be the most accurate means of biological assessment available at present. In the following experiments the staircase effect shown by the myometrium was recorded at varying times after removal of one or more component parts of the reproductive system. In this way it was hoped to assess major changes in hormonal dominance which occur as a result of these interferences.

METHODS

Virgin Himalayan rabbits were mated and 6–10 hr later the right Fallopian tube was ligated under antiseptic conditions, with pentobarbitone and ether anaesthesia. Dettol was the antiseptic employed; it was found that cetrimide and benzylkonium chloride often led to adhesions round the ovary and tube. This operation thus prevented fertilization of the ova on the right side and this horn of the uterus remained non-gravid and available for

recording the staircase effect during pregnancy. On day 24, 25, or 26, taking the time of mating as zero, one of the following operations was performed, again under pentobarbitone and ether anaesthesia: (1) ovariectomy; (2) removal of corpora lutea—the ovaries were subsequently examined histologically to ensure that all luteal tissue had been removed and the results were discounted if removal had been incomplete; (3) removal of gravid horn and ovaries; (4) removal of the foetuses, leaving the placentae *in situ*; (5) removal of the gravid horn; (6) removal of the products of conception.

Between 5 hr and 9 days after this operation one experiment was performed on each animal to record the staircase effect shown by the myometrium. The anaesthetic used initially (Schofield, 1954) was Dial (allobarbitone, Abbot Laboratories), but since the animal appeared to deteriorate somewhat with time, pentobarbitone anaesthesia has been employed in subsequent experiments (Schofield, 1955, 1957).

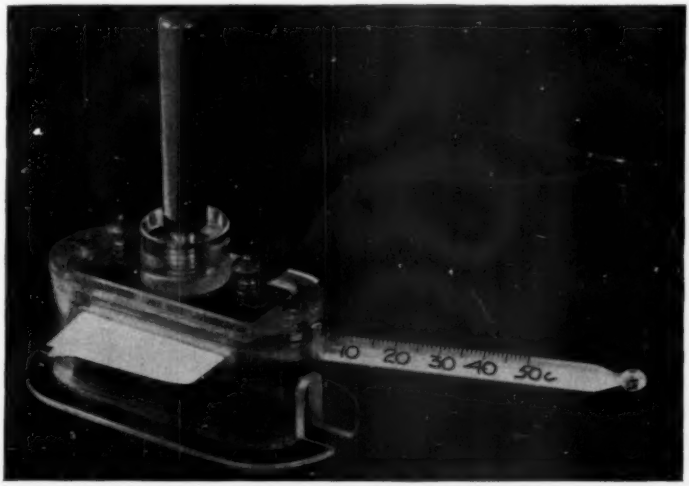


Text-fig. 2. Diagram showing the apparatus for recording the uterine response to direct electrical stimulation.

The technique used for recording the staircase effect was a modification of that used by Bengtsson (1957), which in turn was a substantial modification of the original method (Schofield, 1954). A mid-line incision about 3 in. (7·5 cm) long was made in the abdominal wall and the non-gravid horn brought to the surface. A short length of this horn was then pegged on to the platinum electrodes mounted in Perspex (Text-fig. 2) and secured there with thread which was passed through the holes in the Perspex on either side of the electrode and round the uterine segment. The arms of the electrode attachments *A* and *B* consist of steel rods covered with polythene. The steel is continuous with the platinum electrodes at one end and at the other with a screw for connexion to the stimulator. *A* is carried by a rack and pinion allowing horizontal movement. *B* is so designed that the screw (2) is adjusted to balance the side arm (1), and when *B* is hanging freely the arm (3) is vertical. The side arm (1) is attached by a thread to the short arm of an isometric tension lever which writes on smoked paper (Schofield, 1954). The Perspex chamber which fits over the uterine segment is shown in Text-fig. 3. It is lowered over the uterine segment into the position indicated in Text-fig. 2 so as to bridge over the electrode arms without touching them. The electrode attachments, *A* and *B*, and the Perspex chamber are together supported by an apparatus allowing vertical movement, similar to that described by Bengtsson (1957). The unit is lowered so that the abdominal muscle and skin may be sutured over the flange of the chamber and then, to complete the preparation, raised somewhat to avoid contact between the uterine segment and the abdominal contents.

Environmental conditions were kept as normal as possible by means of a heating coil in

the roof of the Perspex chamber, and below this was inserted a piece of moist filter paper to prevent drying of the uterine segment (Bengtsson, 1957). Temperature was maintained at body temperature and measured by the thermometer (Text-fig. 3). The experimental procedure—adjusting the segment to 'resting length', recording the staircase effect and finding the threshold dose of oxytocin—was similar to that described previously (Schofield, 1957). At the end of the experiment the resting length of the segment was measured (2–3 cm) and the number of foetuses and corpora lutea counted. The ovaries were fixed in Bouin's solution and blocked in paraffin wax. The tension developed by the segment at a frequency of one stimulus per minute was read off the kymograph tracing and expressed as grams tension per centimetre length of segment.



Text-fig. 3. Photograph showing the Perspex chamber which covers the uterine segment.

RESULTS

The normal hormonal pattern of pregnancy (Schofield, 1957) was uninterrupted in only one group of rabbits, namely, that in which the foetuses had been removed leaving the placentae *in situ*. In the remaining five groups a hormonal change-over from progesterone to oestrogen domination occurred after different intervals of time, according to the nature of the operation. The results are best considered according to the groups.

1. *Removal of ovaries.* The staircase effect was recorded between 5 and 66 hr after ovariectomy. The change-over from progesterone dominance appears to take place 7–11 hr after ovariectomy, and thereafter the myometrium is oestrogen-dominated (Table 1). Recordings were made before, during and after abortions, as indicated in the table. The time of abortion after ovariectomy is very variable between 21 and 50 hr after the operation.

TABLE 1. Showing the staircase effects at different times after ovariectomy. One result from each rabbit
B, before abortion; D, during abortion; A, after abortion.

Staircase effect	Hours after operation																																						
	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25		42	43	44	45	46	47	48		66									
Positive								B	B		B	B	B		B		A			D																				
																	D			D																				
																	D			B																				
																	D			B	D		A																	
																	B	B	B	B	B		A		A	D		A												
Transient			B																		B																		
	B		B	B	B	B	B									B																								
			B	B	B	B	B																																	
Negative		B		B	B																																		

2. *Removal of corpora lutea.* The staircase effect was recorded between 7 and 49 hr after these bodies were removed. The change-over in hormonal dominance appears to take place 10–12 hr after the operation (Table 2). As in the previous group, the time of abortion after the removal of the corpora lutea was very variable. It often occurred during the night; in several cases, therefore, it is not possible to state the exact time, and recordings made thereafter may have been several hours later.

3. *Removal of the gravid horn and ovaries.* Recordings were made between 6 and 72 hr after the excision of the ovaries and the gravid horn and are shown in Table 3. The change-over in hormonal dominance from progesterone to oestrogen appears to take place 10–12 hr after the operation.

4. *Removal of foetuses.* The staircase effects recorded some days after removal of the foetuses are shown in Table 4; in all these cases, with the exception of those recorded 32 and 33 days after mating, the placentae were still *in utero*. Some fall in maternal weight and the presence of hair in the cages of these two rabbits indicated that the placentae were lost at about the usual time for parturition.

5. *Removal of the gravid horn.* The recordings were made between 24 and 72 hr after operation and are shown in Table 5. The hormonal dominance appears to change from progesterone to oestrogen 40–50 hr after removal of the gravid horn.

6. *Removal of the products of conception.* The staircase effect was recorded between 20 and 48 hr after the evacuation of the gravid horn. The results are shown in Table 5. They fit in well with those of group 5, since there was no obvious difference in the hormonal effects produced by the two operations.

The ovaries from these last two groups (5 and 6) were examined histologically and compared with control ovaries at the same stage of pregnancy. After removal of the gravid horn or the products of conception, there is degeneration of the corpus luteum; thus Pl. 1, *A* shows a section of the corpus luteum on the 27th day of normal pregnancy and with this may be contrasted the corpus luteum on the 27th day of pregnancy 72 hr after removal of the gravid horn (Pl. 1, *B*). In the latter case degeneration of the corpus luteum is characterized by a diminution in cell size, with resulting irregularities and the predominance of endothelial nuclei, intracellular lipid vacuoles and pycnotic eccentric nuclei. These characteristics are absent from the normal corpus luteum at the same stage (Pl. 1, *A*) and it may be presumed that this corpus luteum is still functional.

It has been shown previously (Schofield, 1957) that when progesterone is the dominant hormone, the myometrium is relatively insensitive to oxytocin, whereas when oestrogen is dominant the myometrium has a

TABLE 2. Showing the staircase effect at different times after removal of the corpora lutea. One result from each rabbit

Staircase effect	Hours after operation																														
	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25		40	41	42	43	44	45	46	47	48	49
Positive							B				B	B		B		B B		B	B	... ...	A		D		A					
Transient				B B		B B														... ...							A A		B B	A A
Negative	B	B B	B		B															... ...										

B, before abortion; D, during abortion; A, after abortion.

TABLE 3. Showing the staircase effect at different times after removal of the ovaries and gravid horn. One result from each rabbit

Staircase effect	Hours after operation																								
	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24		43	44		72
Positive							X X	X		X				X	X				X X X	... ...	X	X	... ...	X
Transient					X											X				...			...	
Negative	X	X	X	X		X X	X													... ...			... ...	

greater sensitivity. To some extent, therefore, the degree of dominance is indicated by the oxytocin sensitivity. Thus a myometrium fully dominated by progesterone, as in mid pregnancy, is completely insensitive. In the experiments reported here, although in those groups showing an early change-over in hormone dominance the oxytocin threshold usually confirmed the hormonal status indicated by the staircase effect, this was not

TABLE 4. Showing the staircase effect recorded at the end of pregnancy after removal of the foetuses on the 24th or 25th day of pregnancy

Staircase effect	Days of pregnancy						
	27	28	29	30	31	32	33
Negative	–						
	–	–	–				
Transient	.	0	0	0			
Positive	+					+	+

TABLE 5. Showing the staircase effect at different times after removal of the gravid horn (H) or the products of conception (P). One result from each rabbit

Staircase effect	Hours after operation																		
	20	21	22	23	24	25		42	43	44	45	46	47	48		66		72	
Positive							...	H	H			P		P	...	H	...	H	
Transient							...	H	P					H	...		...		
							...		H						...		...		
Negative	P				P	H	...		P						...		...		
					P		...								...		...		
					H		...								...		...		

TABLE 6. Showing the oxytocin threshold and staircase effect of the myometrium during the last half of pregnancy in the intact rabbit

Day of pregnancy	Oxytocin threshold (u.)	Staircase effect	Day of pregnancy	Oxytocin threshold (u.)	Staircase effect
16	> 2	–	28	> 2	–
16	> 4	–	28	0·03	–
17	> 4	–	29	0·04	0
17	> 4	–	29	0·2	–
17	> 4	–	29	0·5	–
17	> 4	–	29	0·05	–
18	0·1	–	29	0·05	0
18	> 4	–	29	0·03	–
19	> 4	–	29	0·2	–
20	> 2	–	30	0·02	0
21	> 4	–	30	0·01	0
22	> 4	–	30	0·02	0
23	0·5	–	30	< 0·02	+
24	> 4	–	30	< 0·02	+
24	1·6	–	30	< 0·005	+
25	> 2	–	30	0·2	–
26	0·08	–	31	< 0·005	+
27	0·1	–	31	< 0·005	+
27	> 5	–	31	< 0·02	+

so with a delayed change-over. After removal of ovaries, corpora lutea or ovaries and gravid horn, when the change-over occurred within 13 hr, the average oxytocin threshold for these uteri showing a negative staircase effect was considerably greater than for those showing a positive effect. However, after removal of the products of conception or the gravid horn, when the change-over to oestrogen dominance did not occur till 2 days later, the average oxytocin threshold was the same for the negative, transient and positive staircase effects. In these experiments there is no apparent relation within the different groups between the number of foetuses or the number of corpora lutea on the one hand, and the time of hormonal change-over on the other.

In a previous paper (Schofield, 1957) the tensior developed was considered to provide some indication of the extent of oestrogen influence within the progesterone domination. In this series, however, the tensions developed showed no particular trend which might indicate changes in the influence of oestrogen.

DISCUSSION

The evidence here presented indicates that removal of the foetuses does not greatly affect the progesterone influence, but that the progesterone block is removed, as in the normal course of pregnancy, before the parturition process and that the placentae are delivered in these rabbits at the time usual for the delivery of the foetuses in normal pregnancy. The rabbit thus falls into line with mice, rats and cats (Amoroso, 1956). In these species the foetuses were removed and it was found that the placentae were subsequently delivered at term, as indicated by a change in maternal weight.

The remaining five groups of experiments may be considered under two headings: Group *A*, involving excision of the corpus luteum, which includes those experiments where the corpus luteum itself, the ovary, or the ovary and gravid horn were removed; and Group *B*, involving excision of the placenta, which includes those experiments in which the gravid horn or the products of conception were removed. Group *A* thus represents the influence of the corpus luteum, and Group *B* the influence of the placenta. In Group *A* the time of change-over from progesterone to oestrogen dominance occurs only 12 hr after removal of the corpus luteum, oestrogen being dominant in this series 12 hr after removal of the ovaries and 13 hr after removal of the corpora lutea or ovaries and gravid horn. There is thus no significant difference between the times of hormonal change-over following the three operations in Group *A*; the effect of removing the corpora lutea is apparently not influenced by simultaneous removal of interstitial cells or of placenta. This indicates that the main site of progesterone production is the corpus luteum, but it also suggests that there

is no very significant contribution of progesterone by the placenta as estimated by this method. Hence these experiments appear to demonstrate that in the rabbit the progesterone necessary for the maintenance of pregnancy is produced by the corpus luteum and that this secretion is not substantially augmented by the placenta.

This method of recording from the non-gravid horn ignores any possible local effect of placental progesterone which may be manifest on the myometrium of the gravid horn. In fact Csapo (1956*b*) demonstrated an apparent local effect of progesterone in the rabbit when he found by *in vitro* biological tests that the myometrium at the placental sites showed a greater progesterone dominance than at the interplacental sites. It is possible, however, that the greater dominance was due to a physical concentration of ovarian progesterone at the placental sites, which are very vascular, rather than to an elaboration of progesterone by the placenta itself. Further experiments by Costa & Csapo (1959) also indicate a progesterone influence by the placenta, but this again might be explained by a physical concentration of ovarian progesterone. When the hormonal dominance at the end of pregnancy was followed in the rabbit by the staircase effect, recording on the one hand from a gravid horn (Bengtsson, 1957) and on the other from the non-gravid horn (Schofield, 1957), the time of hormonal change-over from progesterone to oestrogen dominance was found to be similar by the two methods. In the former series the uterine contents were removed immediately before the recording, but it seems unlikely that the effect of placental progesterone on the myometrium would disappear in 1 or 2 hr. The similarity of these results suggests that the staircase effect, whether recorded on the gravid or non-gravid horn, is assessing the influence of ovarian hormones and that the effect of local progesterone on the general behaviour of the myometrium is not very significant. However, it may be that although the ovaries secrete the majority of the progesterone, the placenta does make some minor contribution which can only be demonstrated when the ovarian influence has been removed, as in the experiments of Costa & Csapo (1959).

Considering next Group, *B*, in which the placenta, but not the corpus luteum, was removed, the change-over to oestrogen dominance occurred much later than in Group *A* and was apparent only after 40–50 hr. The time of change-over was similar after removal of gravid horn or products of conception, indicating that excision of one uterine horn has no marked effect on hormonal dominance. This reversion to oestrogen dominance after 2 days suggests either that the placenta secretes some progesterone and when this is removed the progesterone level in the body gradually declines, or that there is some factor present in the placenta which is necessary, either directly or indirectly, for the continued secretion of

progesterone by the corpus luteum. The results in Group *A* have suggested that the placenta makes no very significant contribution of progesterone; the second alternative, therefore, is the more likely and has in fact been considered by other authors (Amoroso, 1955). A study of the histology of the corpus luteum described above shows that after removal of the placenta there are definite signs of degeneration not seen in a control corpus luteum at the same stage. It seems likely, therefore, that the placenta secretes some principle capable of maintaining the corpus luteum, and that when this principle is removed the corpus luteum starts to degenerate and no longer maintains the high level of progesterone which is necessary for a continuance of the stage of pregnancy. Thus the time lag for the myometrium to become oestrogen-dominated would be greater after removal of the placenta, which maintains either directly or indirectly a functional corpus luteum, than after removal of the corpus luteum itself. This would explain the time difference between Groups *A* and *B*, Group *A* being characterized by removal of the corpus luteum and Group *B* by the removal of the placenta.

The correlation of oxytocin threshold and staircase effect which obtains when the hormonal change-over occurs within 12 hr of operation but not when it occurs after 2 days is curious. It indicates that the two tests are not making their assessment on the same property of the muscle. Progesterone influences at least two properties of uterine muscle, 'activation and conduction' (Csapo, 1954). These properties are important for the development of tension in the whole muscle and this was found in the intact rabbit to be less in the myometrium under progesterone domination than in the myometrium under oestrogen domination (Schofield, 1955). The staircase effect is produced by direct stimulation of the myometrium with the electrodes 2–3 cm apart. Obviously the ability of the muscle to respond to a stimulus depends upon the efficiency of conduction between these two points. However, since oxytocin is injected intravenously, it reaches the myometrial cells via the blood stream and has ready access to each individual cell. The property of conduction is not, therefore, so important a consideration where oxytocin sensitivity is concerned. This is one main difference between the two methods of hormone assessment.

When oxytocin sensitivity and the staircase effect were followed through pregnancy in the rabbit (Schofield, 1957) it was found that the myometrium became sensitive to oxytocin 3 or 4 days before the expected date of parturition (Table 6) and that the staircase effect changed only 1 or 2 days before this date. Thus with a declining influence of progesterone, the cells of the myometrium are able to be activated individually before the staircase effect itself shows an altered slope, and this suggests that the progesterone block is removed from the property of activation before that of

conduction. This may account for the discrepancy noted above of the correlation between the staircase effect and oxytocin threshold. Perhaps with a rapid decline in progesterone level, as after removal of the corpus luteum, the staircase effect and oxytocin threshold correlate fairly well, with but a slower decline of progesterone, as after removal of the placenta, or, in the normal animal towards the end of pregnancy, a difference between these two methods of hormone assessment becomes apparent.

It was hoped that these experiments might yield some evidence as to whether the placenta elaborates any oestrogen during the course of pregnancy in this species. Oestrogen is secreted, towards the end of pregnancy at least, by the interstitial cells of the ovary, as can be seen by the follicular development which takes place at this time. However, the time of change-over to oestrogen domination after the removal of the corpus luteum was not significantly altered by simultaneous removal of the interstitial cells when presumably there must be a change in the level of oestrogen influencing the myometrium. The staircase effect is limited in that it indicates only relative amounts of oestrogen and progesterone, not absolute amounts. In fact, it appears only to demonstrate the presence or absence of a dominating progesterone influence, regardless of the level of oestrogen, provided that this is sufficient to maintain the contractile apparatus of the myometrium. These recordings, therefore, do not yield any clues as to the extent and origin of oestrogen secretion. Another indication of oestrogen influence might be the tension developed by the myometrium, which was taken into account on a previous occasion (Schofield, 1957), but these experiments evidently do not range over a long enough period for significant changes in tension, which might be due to a decline in oestrogen, to become apparent.

SUMMARY

1. Virgin rabbits were made unilaterally pregnant by tying one Fallopian tube 6 hr after mating. On day 24, 25, or 26, one of the following was removed—ovaries, corpora lutea, foetuses, gravid horn, ovaries and gravid horn, products of conception. The staircase effect was recorded on the non-gravid horn at different times thereafter in order to demonstrate any changes which might occur in hormonal dominance.

2. It was found that the hormonal dominance changed from progesterone to oestrogen within 2 days in all these experiments except after removal of the foetuses.

3. The experiments can be divided into two main groups: those in which the corpus luteum was removed, when the hormonal change-over occurred within 13 hr, and those in which the placenta was removed, when the hormonal change over occurred after 2 days. This is taken to indicate that the corpus luteum is the main site of progesterone production.

4. Histological evidence indicates that when the placenta is removed the corpus luteum starts to regress and the secretion of progesterone presumably declines. It is concluded that the placenta is contributing some factor which is necessary, either directly or indirectly, for the continued life of the corpus luteum.

5. These experiments indicate that there is no very significant contribution of progesterone by the placenta, though this possibility cannot be completely excluded by the evidence available.

6. After removal of the foetuses, 'pregnancy' appeared to continue normally and was probably terminated at the usual time by loss of the placentas.

7. A correlation between the oxytocin threshold and the staircase effect exists when there is an early change-over in hormonal dominance, but not when this change over occurs after 2 days. This difference is discussed.

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REFERENCES

Amoroso, E. C. (1955). Endocrinology of pregnancy. *Brit. med. Bull.* **11**, 117–125.

Amoroso, E. C. (1956). The endocrine environment of the foetus. *Proc. III int. Congr. on Animal Reproduction, Cambridge.*

Bengtsson, L. Ph. (1957). The endocrine control of myometrial contractility in the uterus of the pregnant rabbit. *Amer. J. Obstet. Gynec.* **74**, 484–493.

Costa, L. M. & Csapo, A. (1959). Asymmetrical delivery in rabbits. *Nature, Lond.*, **184**, 144–146.

Csapo, A. (1954). *Modern Trends in Obstetrics and Gynaecology*, 2nd series. London: Butterworth.

Csapo, A. (1956*a*). Progesterone 'block'. *Amer. J. Anat.* **98**, 273–292.

Csapo, A. (1956*b*). The mechanism of effect of the ovarian steroids. *Rec. Prog. Hormone Res.* **12**, 405–431.

Csapo, A. & Corner, G. W. (1953). The effect of estrogen on the isometric tension of rabbit uterine strips. *Science*, **117**, 162–164.

Schofield, B. M. (1954). The influence of oestrogen and progesterone on the isometric tension of the uterus in the intact rabbit. *Endocrinology*, **55**, 142–147.

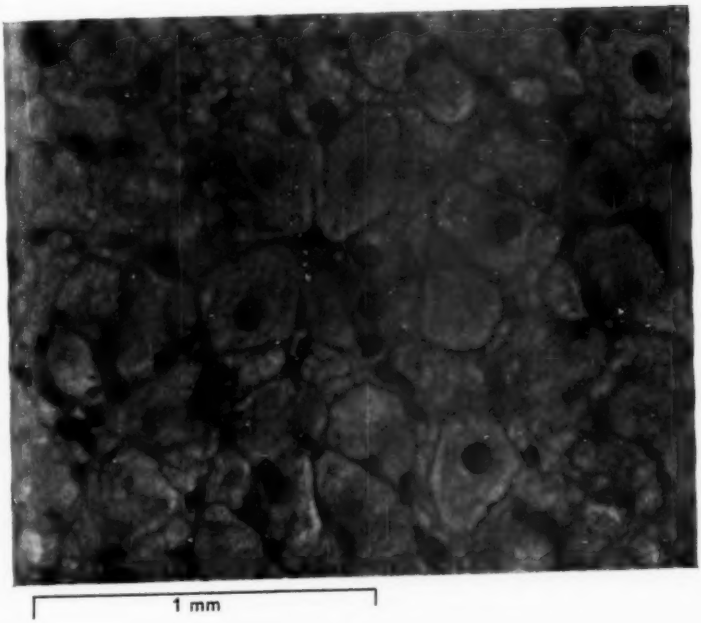
Schofield, B. M. (1955). The influence of the ovarian hormones on myometrial behaviour in the intact rabbit. *J. Physiol.* **129**, 289–304.

Schofield, B. M. (1957). The hormonal control of myometrial function during pregnancy. *J. Physiol.* **138**, 1–10.

EXPLANATION OF PLATE

A. Section of corpus luteum on the 27th day of pregnancy. Cells still functional and regular in size with central nuclei. Lipid vacuoles not obvious.

B. Section of corpus luteum on the 27th day of pregnancy, 3 days after removal of the gravid horn. This is a picture of irregularity with shrunken cells, pycnotic and eccentric nuclei and lipid vacuoles. Endothelial nuclei are predominant. These are all signs of degeneration. Haematoxylin and eosin.



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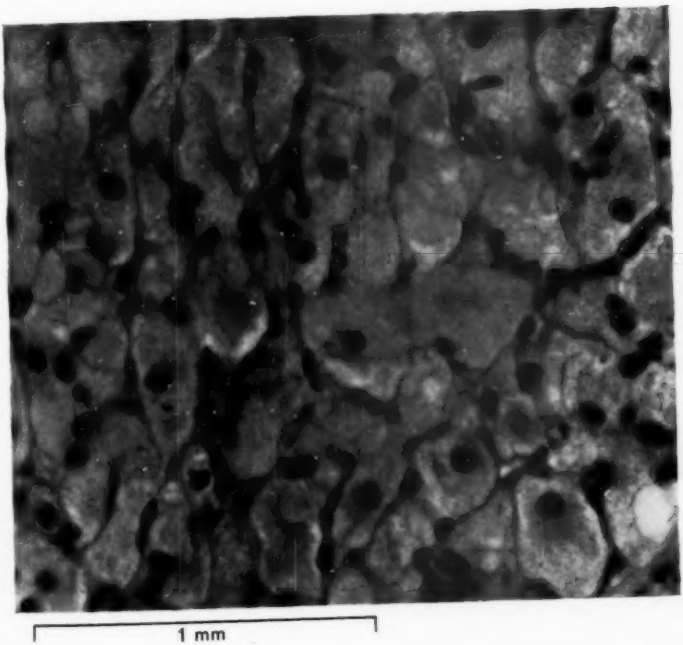

B

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THE EFFECT OF PRESSURE ON SWEATING

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The existence of a hemi-hydrotic effect was shown by Kuno in 1934 (Kuno, 1956), who found that when a man lies on one side there is a remarkable increase in sweating universally over the upper side of the body, very sharply bordered by the median line of the face and of the trunk. This response is not affected by turning the head so that the face is upwards.

Ogata & Ichihashi (1935) considered this effect to be due to posture. It has been attributed by Takagi & Sakurai (1950) to the effect of pressure applied on the skin, even to such pressure as with the finger or the end of a pencil on one side of the body. Watkins (1956), working with West Africans, found frequent changes, of the same order as these, due to chance without any application of pressure. He also tried the effect of applying pressure by leaning against a wall or by pressing on the pectoral region with a cork of 2·5 cm diameter. The occurrence of apparent 'responses' was no more frequent than the chance effects without pressure.

The aims of the experiments described in this paper were to determine whether the hemi-hydrotic effect was due to either pressure or posture. It is difficult to vary one without the other but by using the human centrifuge it was possible to vary the pressure upon the under surface of the body without (*a*) change in posture, or (*b*) applying pressure to any other part of the body.

The investigation can be separated into three parts: (i) quantitative measurement of the hemi-hydrotic effect; (ii) to determine whether moderate pressure (5 kg weight) on the side of the chest would cause a hemi-hydrotic response; and (iii) to determine, by using the human centrifuge, if pressure equivalent to a man's own weight would occasion a hemi-hydrotic response.

METHODS

The subjects were healthy young men, and wore only trousers or shorts.

Quantitative measurement of hemi-hydrotic effect. The subjects lay in a comfortable position on nylon netting (1 cm mesh) which was stretched over a frame to allow free circulation of air. The sweat collection area was not in contact with the netting. The dry bulb temperature varied between 37·8 and 38·5° C; wet bulb temperature between 20·2 and 22·7° C. Air movement was less than 20 ft. (6·1 m)/min. The sweat was measured over the lower ribs in the

mid-axillary line on both sides simultaneously. The sweat was collected on filter paper which was covered by a circular piece of polyvinyl chloride tissue (P.V.C.) attached to the skin by sticking plaster. To keep the filter paper in position and to prevent sweat from outside getting on to the filter paper, a circle of narrow rubber tubing with circumference just greater than that of the filter paper was attached to the under surface of the P.V.C. Slits were made radially in the P.V.C. so that the filter paper could be changed. The flaps so formed were tied in position when the filter paper was inserted.

The filter papers were weighed in weighing bottles before and after sweat collection. The time taken from weighing bottle to position on the skin and vice versa was approximately half a minute. Weighings were made to 10 mg. Each of the two collection areas was 78·5 cm². Collection was continued for 15 min, and an experiment consisted of eight 15 min periods.

To determine whether moderate pressure (5 kg weight on the side of the chest) would cause a hemi-hydrotic response. The subjects sat sideways on a leather seat. Pressure was applied to one side of the chest at the level of ribs 6–10 by means of a sling constructed of a broad band of cloth tapering from 8 in. (20 cm) at the centre to 2 in. at the ends, and attached over a pulley to a 5 kg weight. The subject reversed his position every 15 min, so that the pressure was applied alternately to his right and left sides. The dry bulb temperature varied from 38·9 to 39·2° C; wet bulb from 19·5 to 21·0° C. Air movement was less than 20 ft./min. The method of sweat collection was the same as for quantitative measurement above.

To determine, by using the human centrifuge, if added pressure equivalent to a man's own weight would provide an increase in the hemi-hydrotic response. In this part the whole procedure took place in one car of the human centrifuge (Latham, 1955). The subject lay on a rubber mattress and as during quantitative measurement the lower sweat-collection area was not in contact with the mattress. During each run the car swung so that the direction of the gravitational field was constant relative to the body of the subject. An experiment consisted of four measurements at 1*g*, two measurements at 2*g* and one at 3*g*. The order was: 1, 2, 1, 1, 2, 1, 3. At 1*g*, i.e. with the car stationary, sweat was collected for 7 min; at 2*g* and 3*g* it was collected for 7 min. This latter time was made up of 0·5 min increasing acceleration, 5 min at maximum acceleration, 0·5 min deceleration and 0·5 min each for the application and removal of the filter papers. Dry bulb temperature was $40 \pm 5°$ C.

The sweat collection was made from a much smaller area, 26·4 cm², by using plates similar in shape and size to e.c.g. electrodes and connected across the front and back with rubber bands. A filter paper (diam. 7 cm) was folded round the plate and held in position by another plate. The filter papers were weighed in weighing bottles as before but the use of a more sensitive balance permitted the weighings to be made to 0·5 mg.

RESULTS

Measurements were made, as for quantitative measurement, of the differences in sweat production from upper and lower sides when four subjects lay alternately on their right and left sides. The results are shown in Fig. 1. Each value is a mean of eight observations: thus the upper values are derived from four from the right side when lying on the left, and four from the left side when lying on the right; and conversely for the lower values. The sweat production from the upper side is significantly greater ($P = 0{\cdot}01$) than that from the lower side for all four subjects. Mean values for all four subjects are: upper side 13·0 ml./100 cm²/min; lower side 5·3 ml./100 cm²/min.

The next experiments were designed to test the effect of moderate pressure applied to one side of the chest on the sweat production of the

opposite side. The results are shown in Fig. 2. The values are means of eight, the F_1 means being derived from four from the left side when pressure was applied to the left. The eight values which comprise the F_0 means are in each case taken from the side opposite the F_1 value. None of the four subjects showed any significant difference in sweating between the side to which pressure was applied and the opposite side. Since, however,

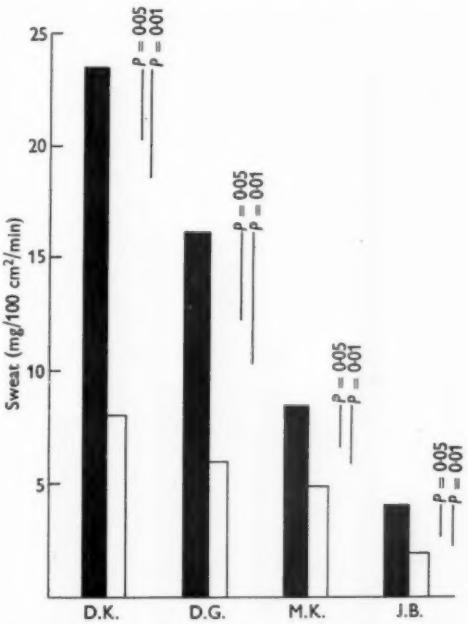

Fig. 1. Hemi-hydrotic effect for four subjects. For each subject sweat production from the upper side (■) is greater than from the lower side (□). Significant differences are given for $P = 0{\cdot}05$ and for $P = 0{\cdot}01$.

this weight (5 kg) is only about 1/14th of that pressing on the under side of a lying man, it does not answer the question of whether Kuno's hemi-hydrotic effect is due to pressure.

In the first experiments both pressure and posture were varied at the same time. In the next, pressure was varied while posture was maintained as constant as possible; nevertheless, some change of posture was unavoidable to counterbalance the effect of the weight. In order to distinguish between the effects of pressure and posture some method was required of applying pressure to one side without having to counterbalance it either by pressure on the opposite side or by change in posture. The human centrifuge was used for these experiments to change the gravitational

field in which the subject lay, and hence the pressure on the lower side of his body. Measurements were made at 1*g*, 2*g* and 3*g*.

The results of these experiments are shown in Figs. 3 and 4. Figure 3 shows the changes with time throughout the experiments; the values are the means of observations on all five subjects. The rate of sweating on the lower side remains constant except for an upward trend which can be

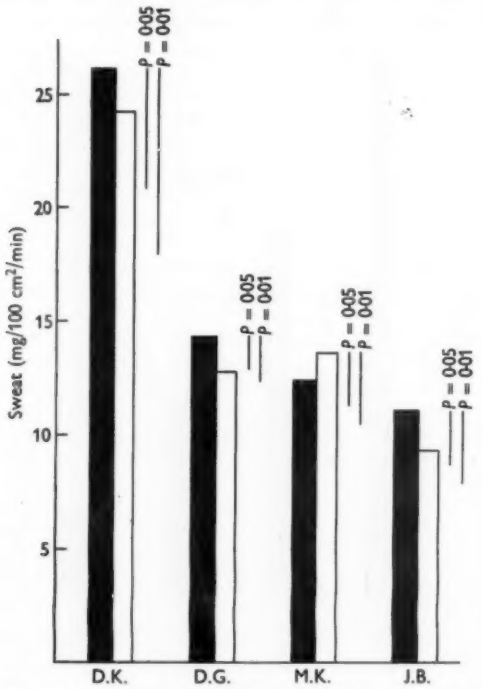

Fig. 2. Lack of hemi-hydrotic response with moderate pressure on one side of the chest in the sitting position for four subjects. □, sweat from side where pressure was applied, ■ sweat from side opposite the application of pressure. Significant differences are given for $P = 0{\cdot}05$ and $P = 0{\cdot}01$.

attributed to warming of the subject. This upward trend is mirrored in the results from the upper side, but in addition there is a greater sweat rate on the upper side at 1*g*; this increase is more marked at 2*g* and still more at 3*g*.

Figure 4 shows the means for upper and lower sides at 1*g*, 2*g* and 3*g* for each subject. For all subjects except P.G., the results are clear. The upper side sweats more than the lower side at 1*g* and this difference is greater at 2*g*. The values given for significant differences apply to 1*g* and 2*g* only; they do not apply to 3*g* because fewer observations were made here. The

results for P.G. were complicated because, while lying on his back, he showed a strong tendency to sweat more on the right side. This tendency has presumably masked the hemi-hydrotic effect at $1g$ when lying on his right side, but the response at $2g$ is significant ($P = 0{\cdot}01$).

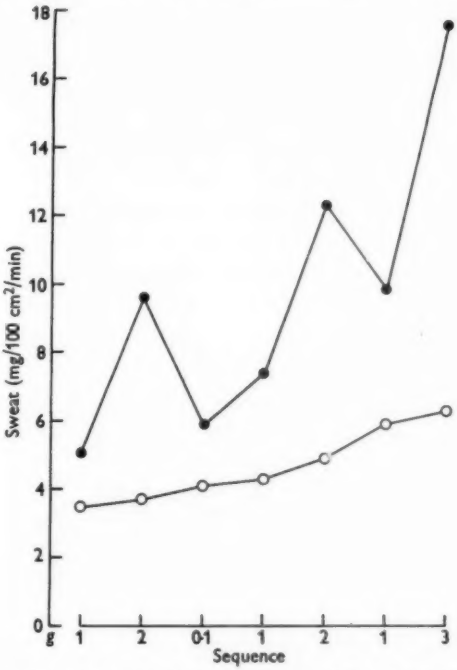

Fig. 3. Diagram showing changes in sweating on the upper side, ●, when the gravitational field was varied, with no changes on the lower side, ○.

DISCUSSION

These experiments failed to show any hemi-hydrotic response to the application of a 5 kg weight pressure on one side of the chest. This is contrary to the conclusions of Takagi & Sakurai (1950), who demonstrated a response to small forces, even when applied with the end of a pencil. Watkins (1956) also failed to show significant responses to small pressures. He measured the sweat rate from the scapular regions on both sides of the body simultaneously without pressure on one side and found that there were spontaneous occurrences of increased sweating on one side with decreased sweating on the other side. The differences were of the same order as the responses to pressure found by Takagi & Sakurai. The same measurements made during the application of pressure on one

side of the body showed no greater frequency of occurrence of such responses. This led to the conclusion that the responses which occurred with pressure were due to chance.

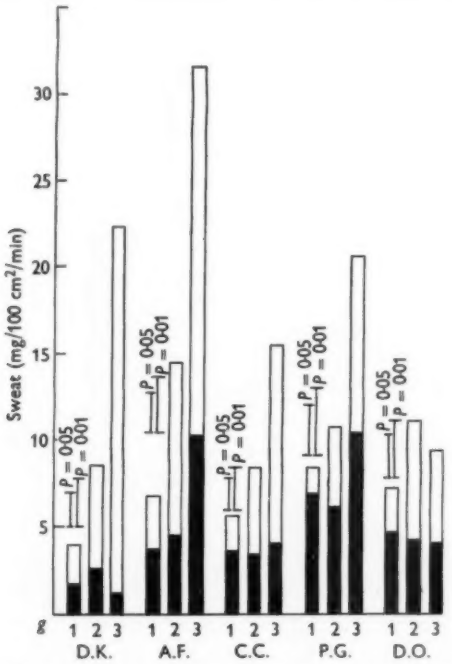

Fig. 4. Hemi-hydrotic response at 1*g*, 2*g* and 3*g* for four subjects; □ upper side, ■ lower side. Significant differences are given for $P = 0{\cdot}05$ and $P = 0{\cdot}01$ for the 1*g* and 2*g* values only.

Ogata & Ichihashi (1935) measured the response in a man lying on his back in a hammock hanging freely and again when it had been turned so that the subject was in an almost lateral position. The usual hemi-hydrotic response was observed when the subject was in the lateral position; but the authors assumed that the 'pressure conditions of the skin surface' had remained unchanged. In addition they found that application of pressure by placing sand-bags on one side of the chest of the supine body produced no hemi-hydrotic effect. They interpreted these two findings as showing that skin pressure was not the cause of the reflex. They then considered whether blood stasis might be the cause, and examined this by suspending or raising the upper limb on one side. Although they showed an increase in sweating on the back of the hand, it was somewhat indistinct in comparison with the cases in which the body was turned.

The conclusions of Ogata & Ichihashi are based on two assumptions: (*a*) that the pressure conditions on the skin surface remained unchanged after rotation of the hammock, and (*b*) pressure applied to the upper surface of the body increases the force acting on the upper surface only. Both these assumptions are incorrect, since in the first case the locus of the pressure has been changed, and in the second, the force acting on the lower surface has been increased to the same extent as that acting on the upper surface. It is considered that their results do not support the conclusion that pressure on the skin is not the cause of the hemi-hydrotic effect.

In the present experiments the force acting on the lower side of the body was increased by doubling or trebling the gravitational field. Since the area in contact with the support increased only very slightly the pressure was also approximately doubled or trebled. No other factor was varied, and hence the increase in magnitude of the hemi-hydrotic effect at $2g$ and $3g$ can be attributed to pressure.

SUMMARY

1. Sweat production was measured by a filter-paper weighing technique from both sides of the chest in the mid-axillary line.

2. When the subjects lay on one side the sweat production on the upper side was greater than on the lower side. This confirmed Kuno's hemi-hydrotic reflex.

3. Application of a moderate pressure (from a 5 kg weight) on the side of the chest when the subject was in a sitting position produced no hemi-hydrotic response.

4. The magnitude of the hemi-hydrotic effect was increased when the subjects lay in a gravitational field of $2g$ or $3g$.

5. The hemi-hydrotic response is due to a change in sweating on the upper side without changes on the lower side.

6. It is concluded that it is pressure and not posture which is the cause of the hemi-hydrotic reflex.

Thanks are due to Dr D. McK. Kerslake for constructive criticism and advice and to the staff of the Acceleration Unit of this Institute for provision of facilities.

REFERENCES

KUNO, Y. (1956). *Human Perspiration*. Springfield, Ill.: Charles C. Thomas.

LATHAM, F. (1955). A man-carrying centrifuge. *Nature, Lond.*, **175**, 1057.

OGATA, K. & ICHIHASHI, T. (1935). Effect of position of the body on sweating. Translated from *J. Orient. Med.* (*Jap.*) **23**, 1127–1154.

TAKAGI, K. & SAKURAI, T. (1950). A sweat reflex due to pressure on the body surface. *Jap. J. Physiol.* **1**, 22–28.

WATKINS, E. S. (1956). The effect on sweating of pressure on the body surface. *Quart. J. exp. Physiol.* **41**, 263–270.

J. Physiol. (1960), **151**, *pp.* 598–607
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SUPERSENSITIVITY OF SKELETAL MUSCLE PRODUCED BY BOTULINUM TOXIN

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In a chronically denervated mammalian skeletal muscle the entire membrane becomes sensitive to applied acetylcholine (ACh). About 4 days after denervation, the size of the ACh-sensitive area at the end-plate starts to increase and a few days later covers the larger part or all of the muscle membrane (Axelsson & Thesleff, 1959).

The conversion of the membrane, following denervation, into an ACh-sensitive surface might be due to the absence of some chemical influence exerted when the motor innervation is intact. The purpose of the present investigation was to see whether or not the release of the chemical transmitter agent might provide such an influence. Use was made of botulinum toxin, which is considered to prevent release of ACh from cholinergic nerve terminals (Burgen, Dickens & Zatman, 1949; Brooks, 1956). This mode of action of the toxin was confirmed, and it will be shown that when the transmitter output was reduced or abolished the size of the receptor area in muscle started to increase in a manner which was identical with that observed after denervation.

METHODS

Unless otherwise stated the experiments were made on the isolated tenuissimus muscle of the cat. The sensitivity of individual muscle fibres to ACh was determined by iontophoretic release of the drug from the tip of a micropipette as described by del Castillo & Katz (1955). When the tip of the pipette was close to the receptor structure, the ACh released by a current pulse of 10 msec duration produced a transient membrane depolarization of a few millivolts amplitude. This potential change was recorded with a conventional capillary micro-electrode inserted into the muscle fibre close to the point of drug application. For details of the experimental set-up, techniques for drug application and recording see Axelsson & Thesleff (1959).

The end-plate region of individual muscle fibres was located by pursuing fine superficial nerve twigs and by the appearance of miniature end-plate potentials (m.e.p.p.s) with a rapid time course. The mean frequency of m.e.p.p.s was calculated from recordings made over several minutes on moving film or with an ink-writer. The part of the muscle membrane at which ACh, when released from a micropipette, produced a depolarization with a latency of less than 10 msec was considered sensitive to the drug. Its length was measured with a binocular dissecting microscope and an eyepiece scale at 80 × magnification.

A powdered preparation of *Cl. botulinum* toxin type A with a mouse LD_{50} of 0·05 μg/kg was used. 1 mg of the toxin was dissolved in 1 ml. of sterile phosphate buffer as described by Ambache (1949). Further dilutions of the toxin were made from this stock solution immediately before use. A fresh stock solution was prepared for each day's experiment.

The toxin, in amounts ranging from 0·01 to 15 μg, was either applied to the exposed surface of the tenuissimus muscle or injected in divided amounts into the musculature of the hind leg. With these doses and modes of administration the action of the toxin was confined mainly to the site of application and generalized intoxications were usually avoided. Two to four weeks after the administration of the toxin the tenuissimus muscle was removed in pentobarbitone anaesthesia. In experiments on frog (*Rana temporaria*) the toxin was injected under the skin of the ventral surface of the thigh.

For examination of the ultrastructure of motor nerve terminals a number of botulinum-intoxicated and control muscles were removed and fixed at 4° C for 1 hr in 1% osmium tetroxide solution buffered with veronal-acetate to about pH 7·5, according to the procedure described by Palade (1952). After fixation the tissue was dehydrated in ethanol and small pieces of tissue containing end-plates were cut out. The specimens were stained in 1% phosphotungstic acid in absolute alcohol and embedded in 'Araldite' according to the method of Glauert & Glauert (1958).

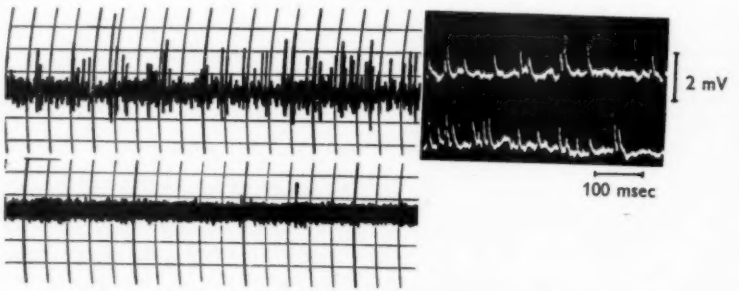
RESULTS

The ultrastructure of motor nerve terminals in botulinum-intoxicated muscles. It has previously been found by R. Thies (personal communication) in acute experiments on isolated serratus anterior muscles of the guinea-pig, that the paralysis produced by bath application of botulinum toxin was not accompanied by structural changes in the presynaptic vesicles. It seemed possible, nevertheless, that chronic intoxication might induce degenerative changes in the nerve endings similar to those described by Birks, Katz & Miledi (1960) during Wallerian degeneration. However, no structural abnormalities were found with the present technique in end-plates from paralysed frog and cat muscles, even after periods of 3–4 weeks. Examples are shown in Pls. 1–3, taken from a frog's sartorius 13 days and from a cat's tenuissimus 27 days after botulinum injection. During the experiments neither of these muscles showed any m.e.p.p.s or responses to nerve stimulation, and the cat muscle had become supersensitive to ACh all along its length. If one compares the micrographs with those from normal end-plates (for frog muscle, see Birks, Huxley & Katz, 1960; for mammalian muscle, see Reger, 1958, and Andersson-Cedergren, 1959), the internal structure of the nerve endings and the relations of their membranes to the muscle fibre and its junctional folds appear to be quite unchanged, nor is there any difference in the size and spatial distribution of the presynaptic vesicles.

The present method would not, of course, reveal changes of a much more minute nature. Thus to demonstrate the location of the toxin molecules or to show up slight changes in the structural detail of the terminal nerve membrane would require a more powerful technique.

Effects of botulinum toxin on transmitter release. As described by Guyton & MacDonald (1947) it was observed that the neuromuscular block produced by a single dose of botulinum toxin reached its maximum in about 5 days and thereafter remained at a constant level for a period of several months.

In amounts exceeding 1 μg the toxin completely abolished transmitter release from motor nerve terminals in the tenuissimus muscle of the cat. In such muscles spontaneous m.e.p.p.s were normally absent and end-plate potentials (e.p.p.s) were not recorded following nerve stimulation.



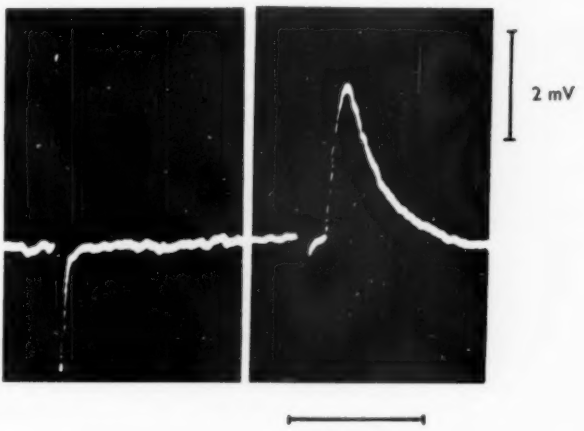
Text-fig. 1. The insertion of the tip of the micro-electrode into the end-plate region of a single muscle fibre gave rise to the burst of m.e.p.p.s shown in the upper ink and cathode-ray oscilloscope recordings. Two minutes later the electrical activity at the end-plate had almost subsided (lower record). The records are from an experiment made on the tenuissimus muscle of a cat which 3 weeks previously had received an injection of 2 μg of botulinum toxin into the hind leg. Neuromuscular transmission was completely blocked and transmitter release was not observed in any other end-plate. Calibration of ink recordings: upper record, 3 squares = 1 mV and 1·5 sec; lower record, 3 squares = 1 mV and 15 sec.

However, the insertion of the micro-electrode occasionally gave rise to a short-lasting burst of m.e.p.p.s at a high frequency of discharge (Text-fig. 1). Apparently this was due to mechanical injury to nerve terminals caused by the tip of the electrode when it penetrated the muscle membrane in the end-plate region.

With smaller quantities of the toxin (0·05–1 μg) m.e.p.p.s with a frequency of discharge about 100 times less than in a normal muscle were observed (mean intervals were of the order of 10–100 sec instead of 0·1–1 sec). In confirmation of earlier investigations (Brooks, 1956) it was found that the amplitude and time course of these m.e.p.p.s were roughly the same as those recorded at a normal end-plate. In these muscles nerve stimulation was either ineffective or produced an e.p.p. of a few millivolts amplitude. At such a relatively light state of intoxication the addition of Ca^{2+} to the bathing fluid in a concentration of twice normal increased the trans-

mitter output by a nerve stimulus (Text-fig. 2). Tetraethylammonium in a concentration of 1 mM had a similar facilitatory action (cf. Koketsu, 1958).

Katz & Miledi (1959) made the interesting observation that in denervated frog muscle spontaneous subthreshold potential changes reappear at the end-plate region several days after cessation of activity. This renewed activity resembled the normal m.e.p.p.s except that the average frequency of discharge was much lower and that the amplitude distribution was wider. Probably end-plate potentials which occur in denervated frog muscle are produced by quanta of ACh released by the Schwann cell, as suggested by Birks, Katz & Miledi (1959).

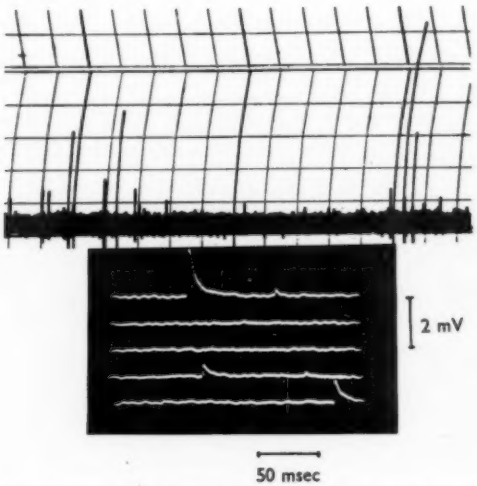


Text-fig. 2. The addition of a twice normal concentration of Ca^{2+} to the bathing fluid resulted in an e.p.p. in response to a nerve stimulus (right-hand record). Before the increase in the external $[Ca^{2+}]_o$ nerve stimulation had no effect (left-hand record).

Since the origin of ACh release in a chronically denervated junction presumably differs from that in an innervated one it was of interest to see whether botulinum toxin would also prevent this type of spontaneous activity. In frogs kept at room temperature the sartorius muscle was denervated on one side and 5 days later 2 μg of botulinum toxin was injected into each leg. This amount of toxin produced a complete paralysis of all leg muscles. Two weeks later the innervated as well as the denervated sartorius muscle was removed, and both were examined for the presence of spontaneous electrical activity at the end-plate region. As shown in Text-fig. 3, a slow rate of spontaneous discharge was observed at end-plate regions in a denervated and botulinum-treated muscle. In the muscle with an intact nerve, however, the same amount of toxin had completely

abolished m.e.p.p.s. Spontaneous ACh release in a chronically denervated junction appears consequently to be less affected by the action of botulinum toxin than the ACh release which in the innervated end-plate originates from motor nerve terminals.

ACh-sensitivity of botulinum-intoxicated muscles. When transmitter release is blocked by the use of botulinum toxin the fibres of the tenuissimus muscle become sensitive to applied ACh along their entire length.



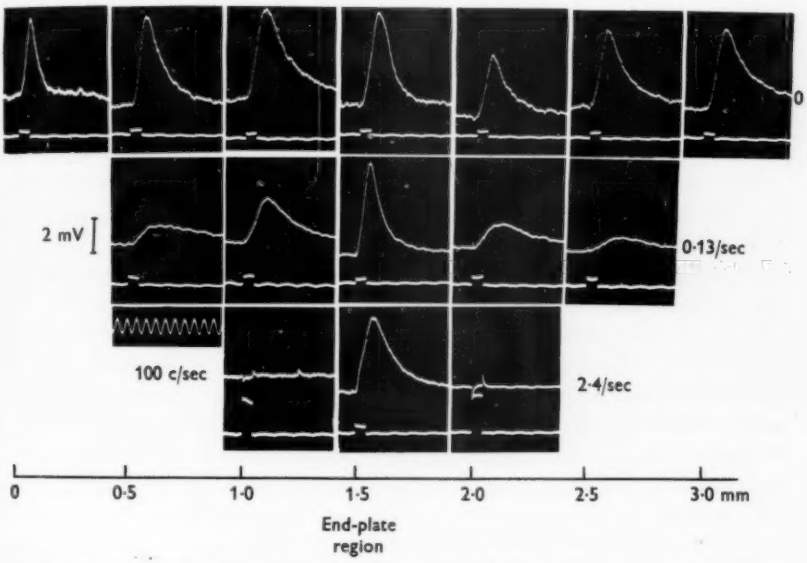
Text-fig. 3. Spontaneous subthreshold activity in a chronically denervated and botulinum-intoxicated frog end-plate (for explanation see text). Calibration of upper record: 3 squares = 1 mV and 15 sec.

One to two weeks after the administration of the toxin, ACh released from the tip of a micropipette produces depolarizations with a rapid time course wherever it is applied to the muscle membrane. The whole surface of the muscle fibre becomes as sensitive to ACh as the end-plate region, which maintains its original responsiveness to the drug (Text-fig. 4). The uniform sensitivity of the muscle membrane to ACh is similar to that which has previously been observed in chronically denervated mammalian muscles (Axelsson & Thesleff, 1959). As in a chronically denervated tenuissimus muscle, ACh produces in a botulinum-intoxicated one a graded and 'electrically silent' contracture.

With a small quantity of the toxin (0·01–0·05 μg) it is possible to obtain tenuissimus muscles in which the ACh-sensitive surface of individual muscle fibres varies in size. This is illustrated in Text-fig. 4. In some fibres the membrane was uniformly sensitive to ACh over at least 3 mm, as is shown in the upper records. In another muscle fibre (middle records) about

2 mm was sensitive to applied ACh, with the highest sensitivity at the end-plate. In a third fibre (lower records) only the end-plate region responded to ACh.

When spontaneous m.e.p.p.s were completely absent or when they appeared at a very low rate (< 0·1/sec) the whole fibre was invariably sensitive to ACh (Text-fig. 4, Table 1). With a higher rate of m.e.p.p. discharge (0·1–1·0/sec) the receptor surface was enlarged as compared to



Text-fig. 4. In a tenuissimus muscle, intoxicated 3 weeks earlier with a small amount of botulinum toxin, muscle fibres were observed in which the ACh-sensitive surface varied in size. The sensitivity of the membrane to ACh was tested by iontophoretic micro-application of the drug. The membrane potential of the fibre is recorded in the upper tracing of each record and the current passing through the pipette in the lower tracing. The fibre used for the upper records was uniformly sensitive to applied ACh over a distance of at least 1·5 mm at each side of the end-plate. The size of the ACh-sensitive surface in two other fibres was smaller (middle and lower records). The frequencies at which m.e.p.p.s occurred are shown by the figures to the right of the records.

normal, whereas at a frequency above 1·0/sec only the end-plate was sensitive to applied ACh. Table 1 shows the rate of m.e.p.p.'s and the mean length of the ACh-sensitive surface in fifty muscle fibres from seven tenuissimus muscles intoxicated 3–4 weeks previously with botulinum toxin. (For technical reasons distances longer than 2 mm could not be measured accurately in single fibres.) The results shown in the table

indicate that, despite individual variations, a relation exists between transmitter release and the extent to which a muscle fibre becomes sensitive to applied ACh beyond the end-plate region.

TABLE 1. The relationship between the frequency of spontaneous m.e.p.p.s and the length of the part of the muscle fibre which was sensitive to applied ACh. The experiments were made on seven tenuissimus muscles intoxicated 3–4 weeks previously with botulinum toxin. Figures within brackets denote the range of measurements

Frequency of m.e.p.p.s (sec^{-1})	No. of fibres	Mean length of ACh-sensitive area (mm)
0·01–0·1	16	2·0 (2·0)
0·1–1·0	8	1·6 (1·0–2·0)
> 1·0	26	0·8 (0·3–2·0)

DISCUSSION

Two observations are of particular interest in the present investigation: first, the finding that botulinum toxin prevents transmitter release from motor nerve terminals without altering their ultrastructure, and, secondly, that this action causes a change in the chemical sensitivity of the muscle similar to that produced by chronic denervation.

It is characteristic of botulinum poisoning that there is a lack of transmitter release from cholinergic nerves, while in other respects nerve and muscle are unaffected by the toxin. The mechanism of this action is not understood, but it is conceivably localized to the nerve terminals (Guyton & MacDonald, 1947; Burgen *et al.* 1949; Brooks, 1954, 1956). The results of the present investigation do not explain the mode of action of the toxin but they suggest, by excluding the possibility of a morphological injury to presynaptic structures, that it is the ACh mechanism in nerve endings which is affected.

The number and size of presynaptic 'vesicles', supposed to contain the quanta of ACh released by the nerve (cf. del Castillo & Katz, 1956), appear to be normal in botulinum-treated muscles. That the terminals still contained packets of multimolecular quanta of ACh was disclosed when a mechanical injury to the end-plate produced a burst of m.e.p.p.s. ACh formation seems to be unaffected by the toxin. Burgen *et al.* (1949) and Stevenson & Girvin (1953) have shown that botulinum toxin does not interfere with choline acetylase systems, and in the present investigation it was observed that spontaneous ACh release from a chronically denervated amphibian junction was unimpaired by the toxin. The results of the present and previous investigations thus indicate that the toxin has a selective mode of action and that its point of attack is the mechanism responsible for the release of the chemical transmitter agent from cholinergic nerves.

In chronically denervated muscles the whole membrane becomes sensitive to applied ACh. This increase in size of the receptor surface can, as was shown by Axelsson & Thesleff (1959), account for the supersensitivity of denervated muscles to ACh and other chemical substances.

In botulinum-intoxicated muscles the spreading of the ACh-sensitive area of the end-plate to the entire membrane occurs in a similar manner and with about the same time course as in denervated muscles. Consequently, it is likely that the cause of the receptor change is the same in both instances. A reasonable assumption is that in denervated muscles, as in botulinum-treated ones, lack of transmitter release and not nerve degeneration is the cause which initiates the increase in size of the receptor surface. That supersensitivity following denervation is caused by the disappearance of transmitter release from the nerve has also been proposed by Burn & Rand (1959), who studied smooth muscles deprived of their sympathetic innervation.

Of interest was the observation that a diminished transmitter release, i.e. a low rate of m.e.p.p. discharge, produced an enlargement of the ACh-sensitive area at the end-plate. This suggests that the size of the chemosensitive region in a muscle fibre is variable and is regulated by the amount and frequency of transmitter released from the motor nerve.

There are no clues as to the nature of the mechanism by which transmitter release might affect the size of the receptor surface in a muscle fibre. A possibility is that the permeability increase produced in the end-plate membrane by the released ACh allows a chemical agent to enter the muscle fibre and that the presence of this substance prevents ACh receptors from being formed outside the end-plate region. Alternatively, the permeability increase may permit the efflux of a substance, formed in the myogenic part of the end-plate, whose presence inside the fibre induces the formation of ACh receptors. This latter possibility could explain the spatial sequence of the change in size of the receptor surface following denervation and reinnervation. This hypothesis is speculative but has at least the advantage that it can be experimentally tested. For example, it might be possible to determine whether long-lasting curarization produces changes in muscle similar to denervation.

SUMMARY

1. In mammalian skeletal muscle intoxicated by botulinum the entire muscle membrane becomes sensitive to applied acetylcholine. One to two weeks after the administration of the toxin the whole surface of the muscle is uniformly sensitive to acetylcholine. The spread of acetylcholine sensitivity from the end-plate to the whole membrane occurs in a similar manner and with about the same time course as in a chronically denervated muscle.

2. Botulinum toxin prevents transmitter release from motor nerve terminals without altering their ultrastructure. The present results indicate that the toxin acts by blocking the mechanism responsible for transmitter release from cholinergic nerve endings.

3. It is suggested that transmitter release from motor nerve terminals determines the size of the acetylcholine-sensitive area in the post-junctional membrane and that lack of transmitter agent and not nerve degeneration is responsible for initiating the process which leads to a high and uniform chemo-sensitivity in chronically denervated or botulinum-treated muscles.

The study of the ultrastructure of motor nerve terminals was made by Professor B. Katz, Department of Biophysics, University College London, who has kindly permitted me to publish his results. The expenses of this investigation were aided by grants from The Muscular Dystrophy Associations of America, Inc. and the Air Research and Development Command, United States Air Force, through its European Office. I am indebted to Dr J. Keppie, of the Microbiological Research Station, Porton, for a generous supply of botulinum toxin and toxoid. Unfailing technical assistance was provided by Miss E. Adler.

REFERENCES

AMBACHE, N. (1949). The peripheral action of *Cl. botulinum* toxin. *J. Physiol.* **108**, 127–141.

ANDERSSON-CEDERGREN, E. (1959). Ultrastructure of motor end-plate and sarcoplasmic components of mouse skeletal muscle fibre. *J. Ultrastructure Res.* Suppl. **1**, 1–191.

AXELSSON, J. & THESLEFF, S. (1959). A study of supersensitivity in denervated mammalian skeletal muscle. *J. Physiol.* **147**, 178–193.

BIRKS, R., HUXLEY, H. E. & KATZ, B. (1960). The fine structure of the neuromuscular junction of the frog. *J. Physiol.* **150**, 134–144.

BIRKS, R., KATZ, B. & MILEDI, R. (1959). Electron-microscopic observations on degenerating nerve-muscle junctions of the frog. *J. Physiol.* **146**, 45–46*P*.

BIRKS, R., KATZ, B. & MILEDI, R. (1960). Physiological and structural changes at the amphibian myoneural junction, in the course of nerve degeneration. *J. Physiol.* **150**, 145–168.

BROOKS, V. B. (1954). The action of botulinum toxin on motor-nerve filaments. *J. Physiol.* **123**, 501–515.

BROOKS, V. B. (1956). An intracellular study of the action of repetitive nerve volleys and of botulinum toxin on miniature end-plate potentials. *J. Physiol.* **134**, 264–277.

BURGEN, A. S. V., DICKENS, F. & ZATMAN, L. J. (1949). The action of botulinum toxin on the neuro-muscular junction. *J. Physiol.* **109**, 10–24.

BURN, J. H. & RAND, M. J. (1959). The cause of the supersensitivity of smooth muscle to noradrenaline after sympathetic degeneration. *J. Physiol.* **147**, 135–143.

DEL CASTILLO, J. & KATZ, B. (1955). On the localization of acetylcholine receptors. *J. Physiol.* **128**, 157–181.

DEL CASTILLO, J. & KATZ, B. (1956). Biophysical aspects of neuro-muscular transmission. *Progr. Biophys.* **6**, 121–170.

GLAUERT, A. M. & GLAUERT, R. H. (1958). Araldite as an embedding medium for electron microscopy. *J. biophys. biochem. Cytol.* **4**, 191–194.

GUYTON, A. C. & MACDONALD, M. A. (1947). Physiology of botulinus toxin. *Arch. Neurol. Psychiat., Chicago*, **57**, 578–592.

KATZ, B. & MILEDI, R. (1959). Spontaneous subthreshold activity at denervated amphibian end-plates. *J. Physiol.* **146**, 44–45*P*.

KOKETSU, K. (1958). Action of tetraethylammonium chloride on neuromuscular transmission in frogs. *Amer. J. Physiol.* **193**, 213–218.

S. THESLEFF

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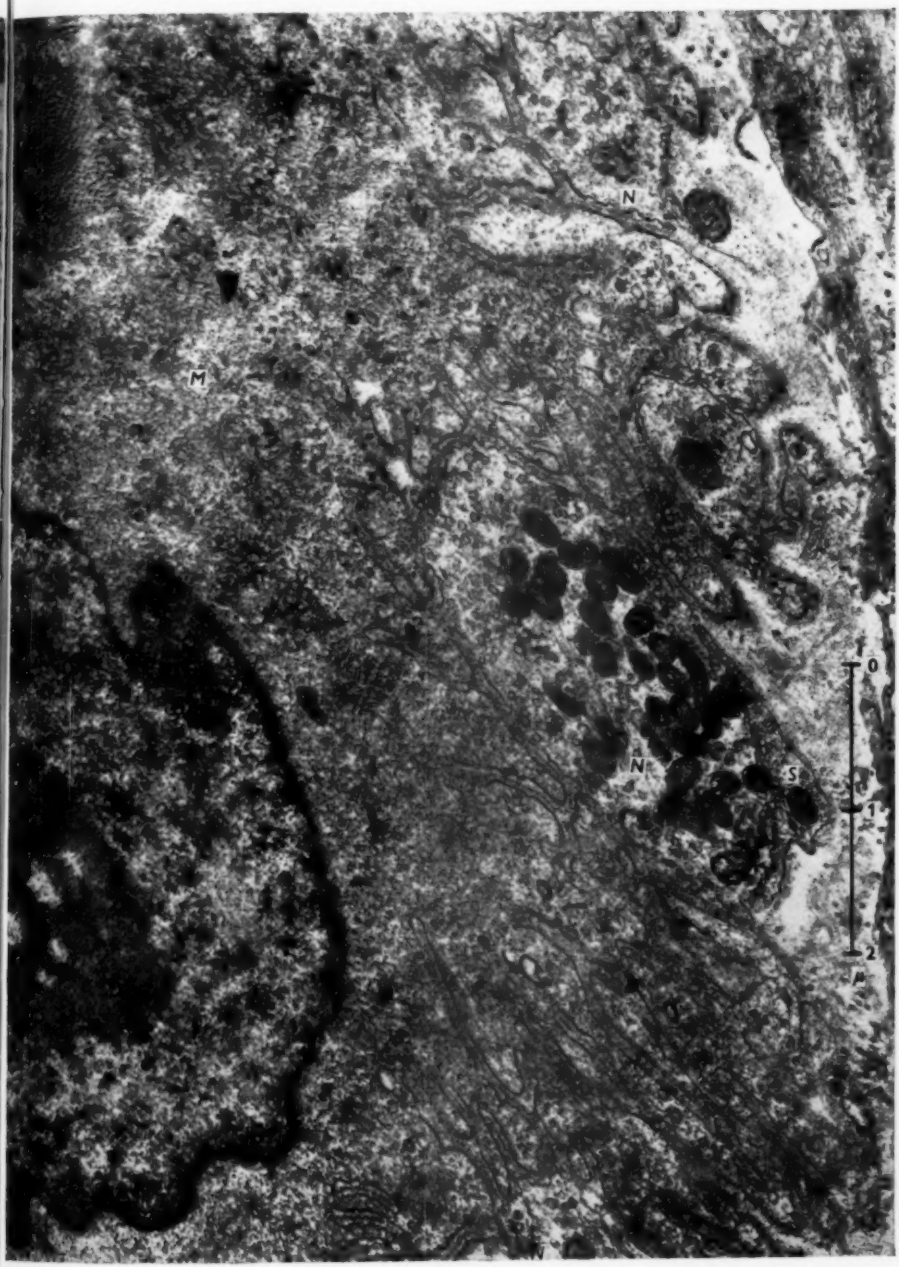

S. THESLEFF

PALADE, G. E. (1952). A study of fixation for electron microscopy. *J. exp. Med.* **95**, 285–298.

REGER, J. F. (1958). The fine structure of neuromuscular synapses of gastrocnemii from mouse and frog. *Anat. Rec.* **130**, 7–24.

STEVENSON, J. W. & GIRVIN, G. T. (1953). The effect of botulinum toxin on the bacterial acetylation of choline. *Atti VI Congr. int. Microbiologia*, **4**, 133–134.

EXPLANATION OF PLATES

Transverse sections of end-plate regions of botulinum-paralysed muscles. Preparations were fixed with osmic acid and stained with phosphotungstic acid in alcohol.

PLATE 1

Frog's sartorius, 13 days after toxin injection. *S*, Schwann cell; *N*, nerve ending; *M*, muscle fibre.

PLATE 2

Cat's tenuissimus, 27 days after toxin injection. *S*, *N* and *M*, as in Pl. 1.

PLATE 3

End-plate from same cat muscle, at lower magnification.

PROCEEDINGS OF
THE PHYSIOLOGICAL SOCIETY

ROYAL VETERINARY COLLEGE MEETING

19–20 *February* 1960

Physiological problems in submarine escape training. By D. E. MACKAY and S. MILES. *Royal Naval Physiological Laboratory, Alverstoke, Hants*

Submarine personnel of the Royal Navy are trained to escape from a sunken submarine with no other apparatus than an inflatable life jacket. When leaving the submarine the air in the lungs is at the pressure of the surrounding water. The man ascends to the surface relaxed and the expanding air, as pressure is reduced, escapes in a prolonged expiration.

Training is carried out in a 100 ft. (30·5 m) water tower at H.M.S. *Dolphin*, Gosport. A scale model of this tower demonstrates the technique and the provision made to minimize the risk of air embolism and to treat it should it occur. It will be realized that any obstruction to the free expansion of air in the whole or part of the respiratory system would produce a pressure in the alveoli above that of surrounding tissue. Where this has occurred air embolism, pneumothorax and retrosternal emphysema have resulted. Such accidents are fortunately rare and 40,000 ascents have been made without a major incident. Records have been made of chest movements during ascent and rates of ascent.

The cuttlefish. By J. B. GILPIN-BROWN. *Marine Biological Association of the United Kingdom, Plymouth* (Film)

A stereotaxic instrument with facilities for checking the alignment of long needle electrodes. By H. C. WRIGHT. *Royal Naval Physiological Laboratory, Alverstoke, Hants*

When single or multiple fine-gauge needle electrodes have to be used for recording brain stem activity, it is an advantage if the alignment of the implanted needles can be checked before the animal is removed from the stereotaxic instrument. It is with this object in view that the instrument described has been designed. The instrument is constructed mainly

with standard components and the amount of machining needed is well within the scope of the workshop of a physiological laboratory. The instrument (without X-ray tubes) is shown in Fig. 1. Both A.P. and

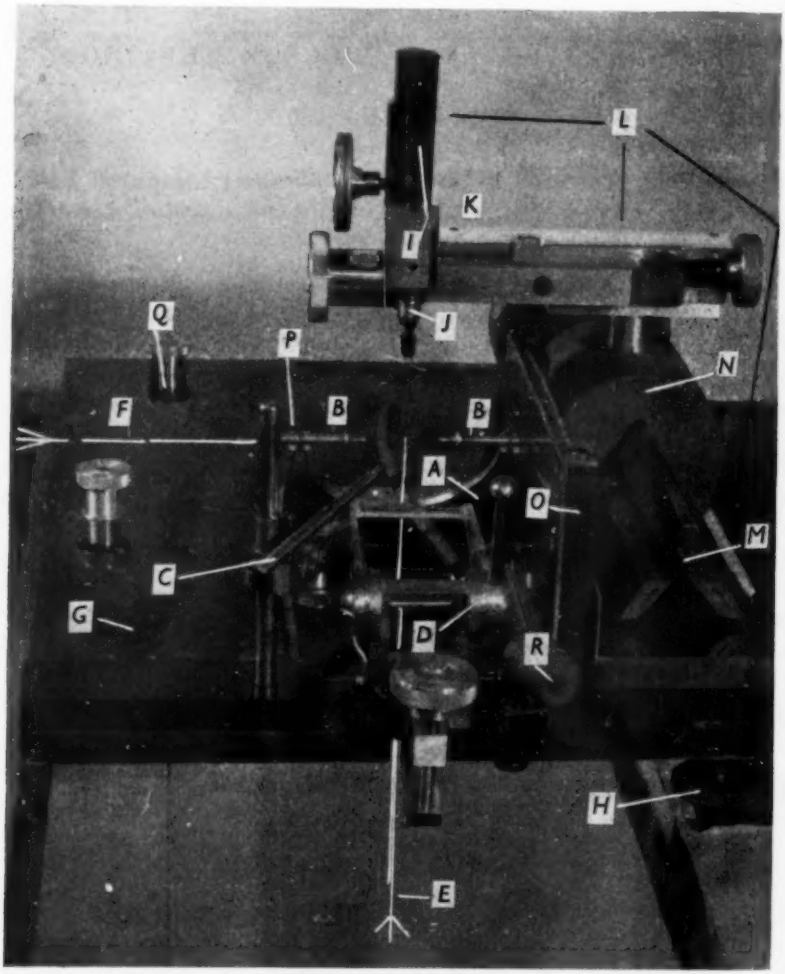

Fig. 1. Key for stereotaxic instrument. *A*, Dural stirrup; *B*, hollow aural pins; *C*, infraorbital probe; *D*, maxillary clamp; *E*, axis of vertical X-ray beam; *F*, axis of horizontal X-ray beam; *G*, adjustable platform; *H*, levelling screws and clamp; *I*, vertical carriage; *J*, detachable holder with needles; *K*, microscope body with coarse rack and pinion movement; *L*, scales for reading co-ordinates (0·5 mm); *M*, triangular base for the carriage; *N*, carriage; *O*, holder for X-ray film; *P*, nylon cords to hold aural pins in stirrup; *Q*, winches to tighten nylon cords; *R*, endotracheal tube.

lateral views of the skull can be taken while the animal is in position. The X-ray tube for the A.P. view is located below the instrument so that the vertical beam passes upwards through the inter-aural axis, the platform holding the stereotaxic instrument can be levelled and also moved transversely (along inter-aural axis) sufficiently to centre the animal's skull in relation to the vertical beam. The X-ray tube for the lateral view is accurately centred by viewing the anticathode (line focus type) through small holes traversing the long axis of the aural pins. The co-ordinates for positioning of needle electrodes are ascertained from a standard cat atlas.

Operation of instrument. The needle (or needles) is mounted on the detachable holder and examined on a travelling microscope with micrometer eye piece for axial alignment. The holder with needle is now mounted on the vertical carriage of the stereotaxic instrument and lowered to the desired position. A control lateral X-ray is taken without the animal. The needles are raised and the animal's head is orientated in the instrument by the aid of two drilled aural pins, the adjustable maxillary clamp and the infraorbital probe. After trephining the skull and reflecting the dura, the needles are lowered to their original position and further X-rays, (A.P. and lateral) are taken of the skull for comparison with the control film. The instrument has been found to be very satisfactory during the last two years.

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The presence of an unidentified pharmacologically active substance in extracts of cerebral hemispheres. By SHIRLEY A. P. PRICE, *Department of Pharmacology, School of Pharmacy, Brunswick Square, London, W.C.* 1

The depolarizing and blocking action of some sympathomimetic amines in the cat's superior cervical ganglion. By D. GOLD and H. REINERT. *Department of Pharmacology, Pfizer Ltd, Sandwich, Kent*

Of the sympathomimetic amines only hordenine was known to have nicotine-like actions in the superior cervical ganglion, the adrenal medulla and other peripheral synapses. It was therefore surprising to find that amines of the amphetamine type produced a nicotine-like stimulation and block in the isolated perfused superior cervical ganglion of the cat (Kewitz & Reinert, 1954; Reinert, 1959).

In recent experiments, with the method described by Paton & Perry (1953), results were obtained which gave conclusive evidence that amphetamines produce depolarization and block in the superior cervical ganglion identical to those described by Paton & Perry (1953) for nicotine. Experiments and results will be demonstrated to show the effects of amphetamines, hordenine and mescaline on the evoked action potential in the superior cervical ganglion of the cat.

REFERENCES

KEWITZ, H. & REINERT, H. (1954). *Arch. exp. Path. Pharmak.* **222**, 311–314.
PATON, W. D. M. & PERRY, W. L. M. (1953). *J. Physiol.* **119**, 43–57.
REINERT, H. (1959). *Proceedings of First International Meeting of Neuro-Psycho-Pharmacology, Rome*, 1958.

A respiratory analyser. By P. SCOTT and K. G. WILLIAMS. *Vickers Research Limited, near Ascot, Berks*

This apparatus measures the dynamic factors in the mechanics of respiration. It is based on transducers which were specially designed to measure pressure and flow in appropriate parts of the respiratory tract and in any associated apparatus. The transducers are used in conjunction with electronic units which convert these respiratory variables into proportional voltages. These voltages can be displayed instantaneously on a cathode-ray tube or other recorder, or they can be computed to give a display which shows more clearly what is happening during the experiment. The voltages can also be recorded on magnetic tape. This means that further analysis of the recordings can be carried out after the experiment has been completed. A low-frequency tape recorder has been specially designed for such use.

The pressure transducers are differential in type and operate as variable capacitances, since they employ a fixed plate and moving diaphragm. The pressure ranges of the transducers are varied by altering the characteristics of the diaphragm. The nominal pressure ranges are $\pm 0{\cdot}5$ cm H_2O, ± 5 cm H_2O, ± 10 cm H_2O, ± 25 cm H_2O and 100 cm H_2O. The chamber volume of the transducer is 0·5 ml. on either side; its entrance is designed to give near critical damping of the output voltage, without excessive overshoot. The capacity changes produced at the transducer alter the frequency of an electronic oscillator and so provide a frequency-modulated signal. A discriminator circuit transforms this signal into a voltage within the range ± 10 V, which is directly proportional to the change in pressure at the transducer.

The flow transducer is of the type which converts flow into a pressure difference. This pressure can then be measured by a sensitive pressure

transducer in the way described above. The transducer element consists of a series of near-parallel plates; the number of plates and the method of measuring the pressure are arranged so that the pressure difference is directly proportional to flow. The ranges of the flow transducers normally used are 0–100, 0–200, 0–300 l./min. In each case the pressure difference at maximum flow rate is 0·5 cm H_2O. The dead space of the flow transducer is only 30 ml. Because the element has a relatively open structure, and is made of water-repellent material, the transducer can be used in both inspiration and expiration without special precautions having to be taken to prevent change in calibration. The transducer is symmetrical and equal pressure differences are produced by a given flow in either direction. This means that ventilatory volume can be measured continuously during pendulum breathing. The pressure difference in the flow transducer is converted into an output voltage (± 10 V) in the same way as in the pressure-measuring channel.

A base-line generator unit gives a zero line dashed at 10 c/s on both the pressure and flow recordings. It does this by rapidly switching the output voltage to zero at a frequency of 2 kc/s. There is a switch to turn this unit off when it is not required.

A high-gain, drift-corrected, d.c. amplifier with capacity feedback is used to integrate the flow voltage. This gives an output proportional to the volume of respired gas passing through the flow transducer. The range of the output can be adjusted to a convenient setting by a scaling switch which alters the input resistance to the amplifier.

A servo multiplier unit generates the product of the pressure and flow measurements. The output from this unit gives the rate of doing work in shifting the respiratory gas. The product is in turn integrated to give a measure of the total work of shifting the gas. The exact meaning of these measures will depend on the positions of the pressure probe and the flow transducer.

All the electronic units are mounted in a rack. The upper unit is used as a control panel. On this panel there are four identical outputs, each with its own switch. The position of this switch determines whether the voltage at that output will be a measure of pressure, flow, volume, work or work rate. This arrangement makes it possible to change from one display to another without disturbing the subject.

We wish to thank the Directors of Vickers Research Limited for their support.

A jerkin plethysmograph for use with the respiratory analyser. By P. J. Heaf and W. D. A. Smith. *University College Hospital, London, W.C. 1, and Vickers Research Limited, near Ascot, Berks*

The measurement of airway resistance with a body plethysmograph has obvious clinical application; unfortunately the method is technically difficult. As an alternative approach, the changes in trunk volume during respiration can be measured by using a double-skinned, air-filled jerkin that covers the thorax and abdomen. Airway resistance can be estimated by comparing these changes with the respired gas volume measured at the mouth with an integrating pneumotachograph.

The jerkin, containing a known volume of gas, is fitted to the subject and the lacing is adjusted to give a positive pressure of 1 cm H_2O at full expiration. During normal inspiration the jerkin pressure rises to 3–4 cm H_2O. These pressure changes are proportional to the changes in trunk volume during respiration and are measured by means of the Respiratory Analyser described in the accompanying communication.

The instantaneous values of thoracic volume and respired gas volume are displayed on the x- and y-axes of a cathode-ray oscilloscope to give a closed loop corresponding to each complete respiratory cycle. By comparing the results obtained with normal subjects with those from patients with airway obstruction, differences in shape of the loops can be seen.

Air temperature and humidity, O_2 uptake and CO_2 output must be considered before absolute values can be given for the mechanical factors of respiration.

We wish to thank the Directors of Vickers Research Limited for their support.

The e.e.g. as an aid to assessment of state of consciousness in the dog. By Phyllis G. Croft. *Department of Physiology, Royal Veterinary College, London, N.W. 1*

Muscle relaxants are being used increasingly in veterinary surgery, and when an animal has been given a general anaesthetic together with a relaxant, it may be difficult to determine whether the animal is unconscious, or conscious but paralysed. In human medicine there have been several reports of patients who have been operated on while conscious, but paralysed by a relaxant (Clark, 1956; Graff & Phillips, 1959; Winterbottom, 1950). There is an obvious danger that this situation may also occur in operations on animals when a relaxant is used at the same time as a general anaesthetic.

The electrical activity of the brain is well recognized as a reliable indicator of the level of consciousness in man and the higher animals. It is, in fact, so closely related to consciousness that a servo-mechanism can be arranged, which maintains a steady level of anaesthesia for 24 hr or more, by using the electrical energy from the brain to operate the plunger of a syringe which is administering the anaesthetic (Wyke, 1958). E.e.g.'s from conscious and anaesthetized dogs indicate that the electrical activity of the brain is a reliable indicator of depth of anaesthesia in this species, too (Swank & Watson, 1949), but lack of a standard technique has so far prevented the establishment of definite criteria for the e.e.g.'s of the various levels of anaesthesia.

Recent work in this laboratory has therefore included the recording of e.e.g.'s from dogs in varying planes of anaesthesia, and from conscious dogs; the e.e.g. can thus be used in future experiments to assess the level of consciousness when relaxants have been given, and the usual reflex tests are no longer valid.

Implanted electrodes, similar to those described by Bradley & Elkes (1953), were used initially, but these were unsatisfactory in some of the dogs. A modification of the type used by Pampiglione, Platt & Stewart (1959) for young dogs and pigs is now being used and proving satisfactory. Hypodermic needles (no. 12, $1\frac{1}{4}$ in. (3 cm) long) are insulated along the shaft with Araldite, and bared at the tip afterwards. They are inserted so that the tips rest on the skull over the anterior one third of the cerebrum on either side of the mid line and about 0·5 cm lateral to it. Leads from a two-channel Ediswan e.e.g. machine are clipped to the butts of the needles. Pentobarbitone sodium or thiopentone sodium is used as an anaesthetic.

Recordings show that the e.e.g. of a conscious dog has a dominant frequency of 25–30 c/s, whilst that of a deeply anaesthetized animal is almost flat with occasional bursts of low-voltage 5–10 c/s activity. Very deep, surgical and light anaesthesia can be distinguished by characteristic e.e.g.'s, dominant frequency and voltage changing as the animal passes from one plane to another. Thus, the e.e.g. can be used to monitor consciousness in man and animals; even when a relaxant has invalidated all the usual reflex tests, e.e.g. records can still provide evidence as to whether or not consciousness has returned. It is hoped that by using the e.e.g. as a criterion other and simpler signs of returning consciousness can be observed and evaluated. An operator could then watch for these signs and so avoid the infliction of unnecessary pain.

REFERENCES

BRADLEY, P. B. & ELKES, J. (1953). *Electroenceph. clin. Neurophysiol.* **5**, 451.
CLARK, D. C. (1956). *Brit. med. J.* ii, 1544.
GRAFF, T. D. & PHILLIPS, O. C. (1959). *J. Amer. med. Ass.* **170**, 269.
PAMPIGLIONE, G., PLATT, B. S. & STEWART, R. J. C. (1959). *Electroenceph. clin. Neurophysiol.* **11**, 603*P*.
SWANK, R. L. & WATSON, C. W. (1949). *J. Neurophysiol.* **12**, 137.
WINTERBOTTOM, E. H. (1950). *Brit. med. J.* i, 247.
WYKE, B. D. (1958). *Brit. med. Bull.* **14**, 58.

A device to facilitate metabolic studies of small restless wild mammals. By A. E. HAWKINS. *Department of Physiology, Royal Veterinary College, London, N.W.* 1

A calorimeter of the Haldane type (1892) is being used to compare the metabolism of small, wild mammals whose restless nature and low CO_2 production make such comparisons difficult. It is possible with the device to be described to collect and weigh the CO_2 produced during several periods of a particular state of activity and to know the total time during which the CO_2 was produced.

The calorimeter shown in Fig. 1 has self-indicating silica gel and self-indicating soda-lime for the collection of water vapour and CO_2 respectively. The gas switch *G* routes the CO_2 from the animal chamber either through weighed collecting U-tubes or through unweighed tubes. This gas switch is shown in detail in Fig. 2. It is made of glass tubing and mounted on a board which can rotate about a pivot *E*. The lever for rotation fits into a gate *A*. In the position illustrated in Fig. 2, the CO_2 from the animal

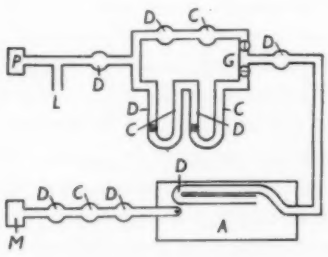

Fig. 1

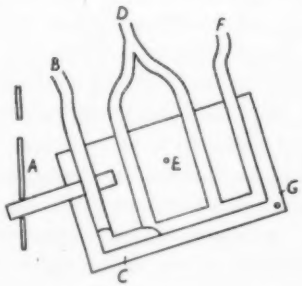

Fig. 2

Fig. 1. Diagram of calorimeter showing position of gas switch. *A*, animal tube; *C*, CO_2 trap; *D*, water vapour traps; *G*, gas switch; *L*, adjustable leak; *M*, air flow meter; *P*, filter pump.

Fig. 2. Diagram of gas switch.

chamber enters through *D*, passes out to the unweighed collecting tubes through *F* and is prevented from reaching the weighed collecting tubes by the mercury *C*.

When the mammal is in the required state of activity the lever is moved to the upper gate position. Consequently, the mercury moves over from left to right so that the CO_2 passes to the weighed U-tubes through *B*, and the peg *G* closes a pair of electrical contacts. An energizing current passes through a relay to the tongue of which is attached a thin, stiff wire. The other end of the wire rests on the balance wheel of a stop-clock. When the relay is energized, the wire lifts and the clock starts. At the end of the period of activity under investigation, the gas switch is turned back to its original position, the CO_2 no longer passes to the weighed collecting tubes and the clock stops.

The apparatus can be used to determine the CO_2 produced during periods of any recognizable stage of activity and it would be possible to use the gas switch and associated timer in a similar manner with instruments to determine O_2 consumption.

The assistance of Mr V. Tindley and Mr F. Gunner in making components of this apparatus is gratefully acknowledged.

REFERENCE

HALDANE, J. S. (1892). *J. Physiol.* **13**, 419.

The contribution of the carotid and vertebral arteries to the blood supply of the cerebral cortex of sheep and calves. By B. A. BALDWIN and F. R. BELL. *Department of Physiology, Royal Veterinary College, N.W.* 1

In the sheep the brain is supplied almost entirely by blood from the carotid arteries. The vertebrals have no direct connexion with the basilar artery, but they have fine anastomoses with the ventral spinal artery. Daniel, Dawes & Pritchard (1953) have shown that in the basilar artery blood flows caudally. In the sheep the arterial input to the intracranial carotid rete through which blood passes before reaching the Circle of Willis is via branches of the internal maxillary artery arising from the external carotid; the internal carotid has no functional significance.

In the calf, in addition to a similar arterial input to that of the sheep, the rete receives a further large component from the basi-occipital plexus formed by the vertebral and occipital arteries. Thus, it appears probable on anatomical grounds that in the calf vertebral blood enters the rete directly before passing to the Circle of Willis, but in the sheep vertebral blood cannot enter the rete directly.

The distribution of dye to the vessels of the cerebral cortex of the sheep and the calf following arterial injection has been examined. Injections of 4 ml. of 1 % Evans Blue or 1 % methylene blue were made in 10 to 12 sec into either the carotid artery at the mid-neck region or into the vertebral artery near to its entrance into the transverse canal of the 6th cervical vertebra. The distribution of the dye to the exposed cerebral cortex was recorded cinephotographically.

The following results were obtained and are shown in the film:

(1) In both sheep and calves injection into one carotid results in coloration of the ipsilateral cerebral hemisphere.

(2) Injection into one carotid with the other carotid clamped results in bilateral distribution of the dye.

(3) In the sheep, dye injected into the vertebral artery does not reach the cerebral cortex. If, however, both carotids are clamped and injection of dye is made into a vertebral artery, the dye is seen in the cerebral cortex, but mainly on the side of the injection. If both carotids and one vertebral artery are clamped, injection of dye into the remaining vertebral artery gives a bilateral distribution.

(4) Vertebral blood normally supplies the cerebral hemispheres in the calf. Injection of dye into a vertebral artery shows a unilateral or bilateral distribution of dye to the cerebral cortex. This variability may be due to variations in the complexity of the basi-occipital plexus.

It can be concluded that, in the sheep and calf, the carotid rete does not facilitate mixing of blood from right and left sides. Vertebral blood plays no part in the supply of blood to the cerebral hemisphere of the sheep, resembling most other species in this respect.

In the calf, on the other hand, a large amount of blood from the vertebral arteries passes to the cerebral hemisphere. In this respect the calf appears to be unique, for in no other species so far recorded does vertebral blood admixed with carotid blood reach the cerebral cortex.

This work was supported by a grant from the Animal Health Trust.

REFERENCE

DANIEL, P. M., DAWES, J. D. K. & PRITCHARD, M. M. L. (1953). *Phil. Trans.* B, **237**, 173.

Modifications to 'The Density Flowmeter'. By B. A. BALDWIN, F. R. BELL and V. C. TINDLEY. *Department of Physiology, the Royal Veterinary College, London, N.W.* 1

The density flowmeter described by Dawes, Mott & Vane (1953) has proved most reliable in our hands, but its value is limited since the range of flows suitable for any given instrument is determined by the size of the

measuring chamber employed. This limitation of a particular instrument to a restricted range of flows has, however, been removed by some minor modifications. If a removable polished Perspex sleeve is introduced into the measuring chamber, a smaller chamber is obtained, suitable for measuring slower rates of flow. If required, the sleeve is introduced before commencing to use the instrument.

The single paired electrodes employed in the original instrument have been replaced by a set of four electrodes, enabling three possible inter-electrode volumes to be selected by means of a switch incorporated in the instrument. This change can be made without difficulty with the instrument in use.

Thus the combination of a removable sleeve and multiple electrodes provides a density flowmeter with six different measuring-chamber volumes incorporated in a single instrument.

We have used instruments with the following dimensions:

	Measuring-chamber volume	
Different electrode pairs	With sleeve (ml.)	Without sleeve (ml.)
V1	2·41	8·93
V2	4·76	17·86
V3	7·25	27·88

A single instrument of these dimensions has been used to measure blood flows of from 10 to 500 ml./min.

REFERENCE

DAWES, G. S., MOTT, J. C. & VANE, J. R. (1953). *J. Physiol.* **121**, 72.

A dual-range thermistor thermometer for field or laboratory work.

By F. H. GULSON, J. T. GUNNER and V. C. TINDLEY. *Department of Physiology, Royal Veterinary College, London, N.W. 1*

The thermistor (Rosenberg, 1947) used as a resistance thermometer has biological applications where there is a requirement for remote indication of temperature, small size of sensitive element and a rapid response to changes in temperature (Andrews, 1947).

With the self-contained robust 25–0–25 microammeter the thermistor thermometer was designed for taking temperatures of small mammals or 'cold-blooded' animals in field work to an accuracy of 0·05° C. In the laboratory a Pye Scalamp galvanometer can be switched in to give 0·01° C for 1 mm deflexion (maximum sensitivity on the 'direct' range is 40 cm/1° C). A fixed value wire-wound resistor 'shunts' the thermistor, thus giving a linear response instead of the natural exponential one of the

thermistor alone. This allows a greater range of temperatures to be easily measured. If suitable thermistors are chosen and 'shunted' to give the same response slope, this method can be used to give multiple ranges with direct reading, by using the existing pre-set points of the instrument. In a prototype instrument, with two thermistor probes, which is being used for taking the anal temperature of bats in connexion with an anti-rabies field research project in the West Indies, a range of 0–20, 20–50° C was

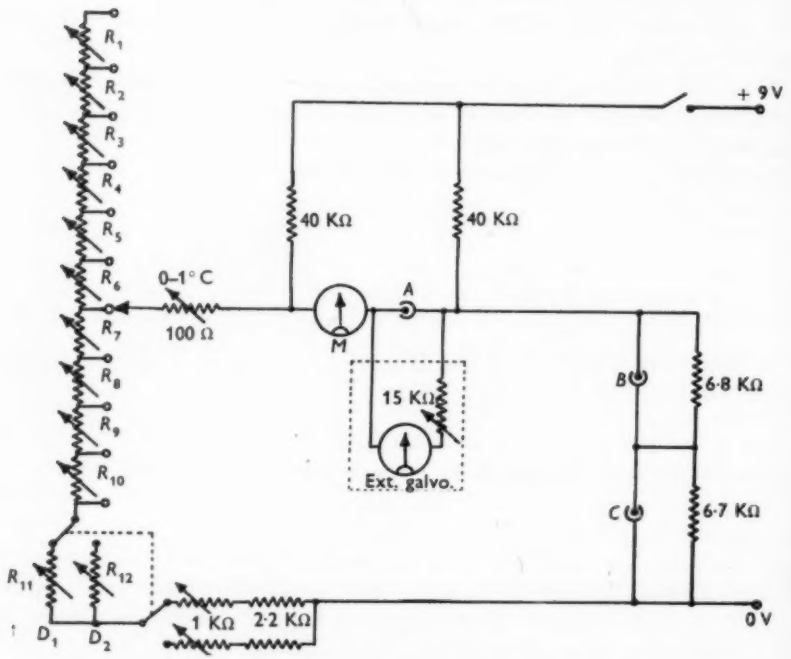

Fig. 1. Sockets *A*, *B* and *C* are closed circuit types. M = 25–0–25 microammeter; R_1 to R_{12} are 100 Ω pre-set types; B = 32–42° C thermistor socket; C = 15–25° C thermistor socket.

covered with 0·1° C accuracy by using 'shunted' thermistors. These thermistors were made in the laboratory. However, we have since found that the Standard Telephone Co. will supply very similar thermistors, and it is their 'A' type unmounted thermistors that we have used in our present instrument.

The usual Wheatstone bridge circuit (Fig. 1) is used with pre-set wire-wound resistors in the 'balance' arm. A displacement resistor for

each range, (D_1, D_2), enables small random changes in thermistor characteristics to be compensated. We find the inherent stability of these thermistors to be of the order of a few hundredths of a degree in any 24 hr period but, in the course of a month, the variation may be 0·1° C or even 0·2° C. Since the sensitive external galvanometer (Pye Scalamp) has a total resistance of about 13,000 Ω, only a negligible fraction of the total current flows in the galvanometer circuit for small deflexions, and hence, for a small range (0·5° C either side of centre zero), the galvanometer deflexion is directly proportional to our linear temperature response. With a suitable series resistor, the galvanometer deflexion can be adjusted to a convenient scale, i.e. 1 cm = 0·1° C, and temperatures can then be read directly from the galvanometer scale with an accuracy of at least 0·01° C. All the resistors used were of the wire-wound type and with the same small positive temperature coefficient in order to cancel out any 'balance' disturbances due to variations of ambient temperature.

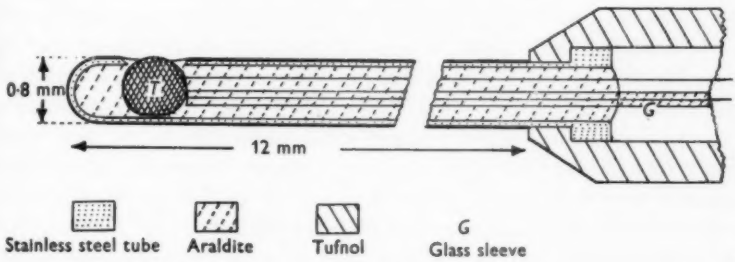

Fig. 2.

The thermistor bead (T, Fig. 2) is inserted through a hole in the side of a 0·8 mm external diam., closed-end, stainless-steel tube. A glass sleeve (G) insulates one of the 0·001 in. (0·0025 mm) diam. wires from the other, and the assembly is sealed in position with Araldite. About 25 % of the surface area of the bead, which is flush with the external surface of the stainless-steel tube, will be in actual contact with the tissues of the animal (except for a thin protective coating of Araldite varnish). This device permits a response time of less than a second. To prevent heat being absorbed by the metal tube from the surrounding tissue, a thick coat (0·005 in. 0·013 mm approx.), of Araldite varnish was put on the external surface of the metal tube.

REFERENCES

ANDREWS, B. L. (1947). *Electron. Engng*, **19**, 288.
ROSENBERG (1947). *Electron. Engng*, **19**, 185.

Microchemical identification of progesterone. By E. G. C. Clarke and Sheila Taylor. *Department of Physiology, Royal Veterinary College, London, N.W. 1*

Progesterone is usually identified by its ultra-violet absorption spectrum, determined after counter-current distribution (Pearlman, 1954) or paper chromatography (Zander & Simmer, 1954); but as Short (1958) points out, this criterion is not really specific and does not serve to differentiate between progesterone and certain other steroids. It thus seemed that there was a definite need for a simple chemical test for progesterone which would combine high specificity with a sensitivity in the microgram range.

Identification of compounds from the appearance of microcrystalline derivatives, a technique well known in alkaloidal chemistry, does not appear to have been applied to steroids, and seemed worth investigating. Barger & Field (1912) recorded a blue compound of iodine and cholic acid, while Zaffaroni, Burton & Keutmann (1950) noted a similar reaction between cortisone and iodine. Bassil & Boscott (1951) found that this cortisone-iodine complex, which was partially crystalline, was only stable in the presence of excess iodine, being dissociated by excess potassium iodide. Our experiments have shown that progesterone forms a similar complex with iodine, but in this case the compound formed in the presence of excess iodine is unstable, while that formed in the presence of excess potassium iodide crystallizes in reproducible form.

The test is carried out with the hanging microdrop technique developed by Clarke & Williams (1955). A microdrop (0·1 μl.) of a solution containing 1 g of iodine and 50 g of potassium iodide in 100 ml. of water (Fulton, 1929) is placed on a glass cover-slip and a microdrop of an ethanolic solution of progesterone (which may have been eluted from a paper chromatogram) is added with slight stirring. A cavity slide ringed with gum arabic solution is inverted over the cover-slip, then re-inverted and examined under low magnification. Progesterone gives irregular rosettes of fine needles which polarize brightly. The crystals form in a few minutes from a 1/1000 solution, but need several hours in a 1/10,000 solution.

Several of the forty other steroids tested give crystals under these conditions, but none of them resemble those given by progesterone. Of the compounds in the pregnane series, for instance, 5β-pregnane-3,20-dione gives fine branching needles, deoxycorticosterone gives triangular plates and 3β-hydroxypregna-5,16-dien-20-one gives stout needles.

Our thanks are due to Dr W. Klyne for kindly providing us with steroids from the Medical Research Council Steroid Reference Collection.

REFERENCES

BARGER, G. & FIELD, E. (1912). *J. chem. Soc.* **101**, 1404.
BASSIL, G. T. & BOSCOTT, R. J. (1951). *Biochem. J.* **28**, xlviii.
CLARKE, E. G. C. & WILLIAMS, M. (1955). *J. Pharm., Lond.*, **7**, 255.
FULTON, C. C. (1929). *J. Ass. off. agric. Chem. Wash.* **12**, 315.
PEARLMAN, W. H. (1954). *Recent Progr. Hormone Res.* **60**, 27.
SHORT, R. V. (1958). *J. Endocrin.* **16**, 415.
ZAFFARONI, A., BURTON, R. B. & KEUTMANN, E. H. (1950). *Science*, **3**, 6.
ZANDER, J. & SIMMER, H. (1954). *Klin. Wschr.* **32**, 529.

Pace-maker functions of giant cells, and large fibre conduction in the pallial nerves of *Archachatina*. By R. H. NISBET. *Division of Biology, Royal Veterinary College, London, N.W.* 1

As previously reported (Nisbet, 1957) the giant cells in the ganglia of *Archachatina* are associated with 40–60 μ neurones. Each giant cell gives rise to a neurite (Bennett, Crain & Grundfest, 1959) from which several large (10–30 μ) collaterals arise, at least one of which divides into fine collaterals in neuropil adjacent to small (15–20 μ) neurones. Other collaterals leave the ganglia in the visceral and pallial nerves.

Responses of the ganglion cells to centripetal stimulation (either tactile at the collar or electrical to a pallial nerve) differ from those of *Mya* (Horridge, 1958) in several respects.

Tactile stimulation is followed by reflex bursts of activity with a minimal latency of 25–30 msec, and frequently this is much longer. The activity evoked is of relatively short duration, declining in 0·5–1·5 sec. Single, maximal (*c.* 400 mV) electric shocks (rectangular, duration 1–2 msec, at 0·7 sec intervals) evoke efferent responses which are small and regular in form, with latencies of 15–30 msec. Short bursts of repetitive stimulation (32/sec for 1–2 sec) of similar strength lead to a cessation of response. This can be overcome by increasing the stimulus strength to 5 V, when small potentials (up to 200 μV) appear after a latency of 60–100 msec and larger compound potentials (0·5–1·0 mV) after 700–750 msec. The large groups synchronize to each third stimulus, but in a few seconds accommodation occurs, the large potentials cease and the smaller potentials decrease in number. On raising the stimulus to 10 V or more the large potential groups reappear in 90–120 msec and rapidly synchronize with each stimulus, the resulting activity continuing for many seconds or even several minutes.

In the nerves, decremental conduction (Schlote, 1955) is being examined with special reference to the large fibres. By means of threshold stimula-

tion it has been established that the action potentials of fast conducting fibres undergo a decrement, but this can be due to two different causes.

In the visceral nerve (Fig. 1), which contains many ganglion cells, the fast fibre potential undergoes changes in form, at 27–30 mm from the abdominal ganglion, which imply that the moving electrodes are recording the exponential decay of a post-synaptic potential.

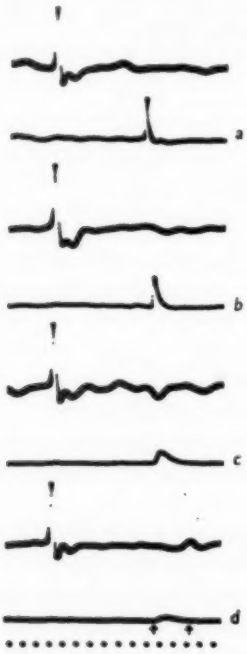

Fig. 1

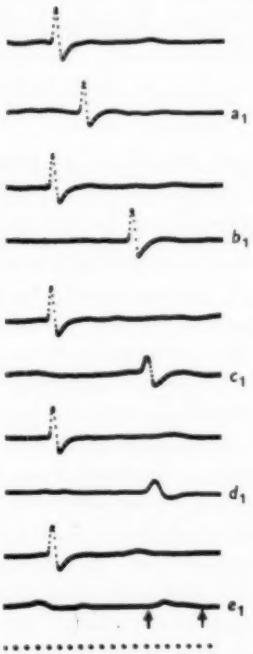

Fig. 2

Figs. 1, 2. *Archachatina*: decremental conduction in large fibres. Upper beam: marker potential; lower beam: potential at successive points along the nerve. Electrode pairs 0·75 mm apart. Time: 2 msec. Temperature: 22–23° C. Fig. 1. Visceral nerve. Marker (1·2 mV) at 10 mm from ganglion. Potential (*a–d*) at 27, 28, 29 and 30 mm from ganglion. Average velocity 115 cm/sec. Gain difference between upper and lower beams 10:7. Fig. 2. Anterior fine right pallial nerve. Beam modulation at 10 kc/s. Marker (1·0 mV) at 12 mm from ganglion. Potential (a_1 to e_1) at 17, 27, 30, 31 and 32 mm from ganglion. Maximum velocity 125 cm/sec. At c_1 and e_1 velocity was 100 and 50 cm/sec. Rise time and duration before decrement, 0·5 and 1·2 msec. Arrows indicate smallest measurable potentials.

In a pallial nerve (Fig. 2), although the action potential follows a similar course at 28–32 mm from the parietal ganglion, there is in this case a steady decline in velocity (125 cm/sec to 50 cm/sec), a slowing of the rise time,

an increase in duration and finally the assumption of a compound form with duration 8–10 msec. This pattern suggests that the large collateral has divided into a number of fine fibres before reaching the level of peripheral radiation.

The foregoing results pose a number of questions with respect to the possible pacemaker function of the giant cells (Arvanitaki, 1942), their relationship to the other neurones in the ganglia and the distribution of their large collaterals.

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REFERENCES

Arvanitaki, A. (1942). *J. Neurophysiol.* **5**, 89–108.
Bennett, M. V. L., Crain, S. M. & Grundfest, H. (1959). *J. gen. physiol.* **43**, 159–250.
Horridge, G. A. (1958). *J. Physiol.* **143**, 553–572.
Nisbet, R. H. (1957). *J. Physiol.* **135**, 34–35*P*.
Schlote, F. W. (1955). *Z. vergl. Physiol.* **37**, 373–415.

Physiological adaptation to environment in the *Littorinidae*. By P. A. Mayes. *Department of Physiology, Royal Veterinary College, London, N.W.* 1

The four British species of periwinkles, *Littorina neretoides*, *L. saxatilis*, *L. littorea* and *L. littoralis* occupy characteristic zones in the intertidal region of the rocky shore (Colman, 1933). Species farthest up the shore and washed less by the sea have to withstand the greatest variation in external salinity resulting from drying, on the one hand, and dilution by rain, dew and mist on the other. Experiments were carried out to evaluate the importance of variations in salinity on the zonation of *Littorina*.

Specimens were equilibrated in sea water (salinity 35·5 g/kg), then immersed in solutions of differing salinity and weighed at intervals (Fig. 1). *L. neretoides* survived and showed locomotion in a solution having twice the salinity of sea water. On the other hand, *L. littoralis* did not survive beyond 24 hr in this solution. In 50 % sea water (v/v) all animals increased in weight reaching a constant level, the magnitude of which varied between species in a manner reflecting their zonation. In 25 % sea water, all species remained retracted in their shells and died ultimately in a swollen condition. The time of survival in very hypotonic solutions appeared to depend on the efficiency with which the operculum fitted the shell. Apparently sea water trapped within the confines of the shell on closure of the operculum could act as an equilibration chamber between the body fluid and the external medium.

It seems likely, therefore, that salinity variations could play a part in determining the zonation of the Littorinidae. Of the species studied *L. neretoides* is the most independent of changing salinity and inhabits

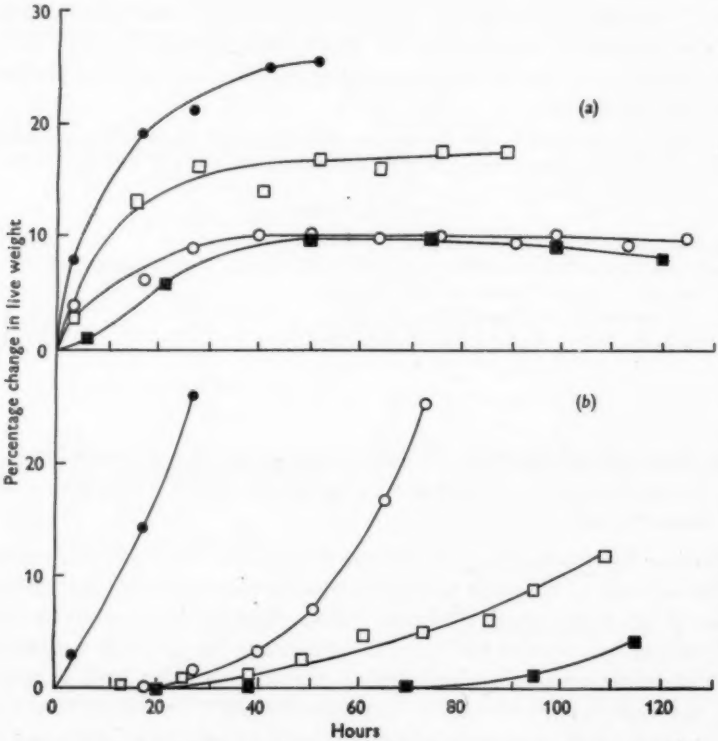

Fig. 1. The effect of (*a*) 50% sea water, and (*b*) 25% sea water upon the weight regulation of *Littorina*. Each point is the mean of seven animals. Before weighing they were carefully drained and superfluous fluid absorbed with filter paper. Live weight = total weight minus shell weight. ●, *L. littoralis*; □, *L. littorea*; ○, *L. saxatilis*; ■, *L. neretoides*.

the splash zone of maximum salinity fluctuation. Conversely, the species requiring a minimum variation in salinity (*L. littoralis*) exhibits the most rigid zonation, being confined under the fronds of the brown algae of the middle shore.

REFERENCE

COLMAN, J. (1933). *J. Mar. biol. Ass. U.K.* **18**, 435.

Gonad changes in the wild red fox (*Vulpes vulpes crucigera*). By R. F. S. Creed. *Division of Histology, Royal Veterinary College, London, N.W.* 1

The only report on reproduction in the red fox in Britain known to me is that of Rowlands & Parkes (1935). This was carried out on a small number of animals consisting of ten males and eighteen females. All except three of these were obtained in their first year as cubs and kept in captivity for varying periods up to 3 years. These animals failed to breed.

In the present study, 137 adult males and 183 adult females have been investigated. The animals were shot or otherwise killed in the field during the years 1955–57 and the majority were transported as soon as possible to the laboratory. The remainder were dissected in the field. The genital tracts of both sexes were dissected out, weighed and measured. The ovaries of the females and the testes and epididymides of the males were then removed and weighed separately and pieces of these tissues were prepared for histological examination.

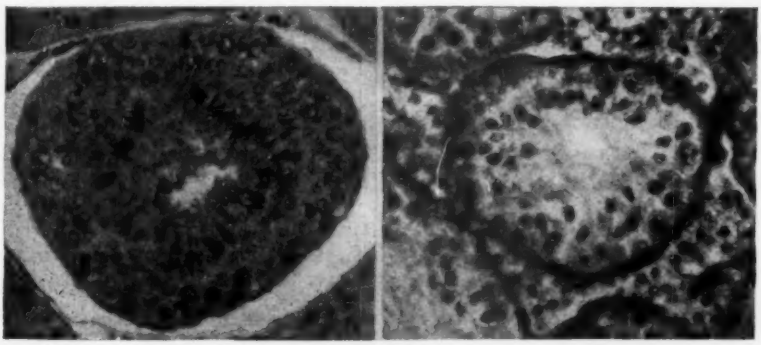

Fig. 1 Fig. 2

Fig. 1. T.S. Seminiferous tubule of fox killed in February showing complete spermatogenesis. × 440.

Fig. 2. T.S. Seminiferous tubule of fox killed in August showing limited spermatogenic activity. × 440.

Examination of ovarian weights throughout the year reveal considerable seasonal variation. During the months of June, July, August and September each pair of ovaries weighs between 250 and 400 mg. During October, November and December and, in some cases, January, the ovarian weights increase progressively, reaching between 600 and 700 mg. This is followed by a well marked and rapid increase during January, February and early

March when a final weight of 1000 to 2500 mg is attained. A gradual decline then occurs during the later part of March, April and May.

Histological examination shows the initial increase to be due to follicular development, and the large and rapid increase in January and February to be the result of the formation of corpora lutea following ovulation. The subsequent decline is due to the regression of the corpora lutea which, however, are recognizable for a considerable time after parturition.

Examination of paired testis weights reveals a comparable seasonal variation, maximum weights of approximately 12,000 mg being attained in January and early February. A gradual decline occurs in March, April and May and in June, July, August and September, the weight of the gonads averages approximately 2500 mg. This is followed by progressive increases during October, November and December. These increases in testis weight are primarily due to an increase in size of the germinal epithelium.

During the period May to August the testes show early stages of spermatogenesis only (Fig. 2), whereas, in January and February, the testes show full spermatogenic activity (Fig. 1).

In the epididymides the pattern of activity is similar to that shown by the testes. Thus, in the wild red fox, the males and females reach full breeding condition only in January, February and early March.

REFERENCE

ROWLANDS, I. W. & PARKES, A. S. (1935). *Proc. zool. Soc. Lond.* p. 823.

An animal preparation for the investigation of the physiology of the normal portal venous circulation. By F. R. BADRICK and C. SHALDON. *Department of Surgery, Bristol Royal Infirmary, Bristol*

Electron microscopy of the gastric mucosa. By A. M. LAWN. *Department of Physiology, Royal Veterinary College, London, N.W.* 1

The metabolism of small wild animals. By A. E. HAWKINS and P. A. JEWELL. *Department of Veterinary Physiology, Royal Veterinary College, London, N.W.* 1

An apparatus for recording the spontaneous activity patterns of small mammals. By A. E. HAWKINS. *Department of Veterinary Physiology, Royal Veterinary College, London, N.W.* 1

Some factors affecting the histidine decarboxylase activity of tissues. By J. M. TELFORD. *Department of Pharmacology, School of Pharmacy, London W.C.* 1

The goitrogenic effect of calcium carbonate on the rat contrasted with its iodine-sparing effect on the cat. By M. G. SCOTT and PATRICIA P. SCOTT. *Royal Free Hospital School of Medicine, London, W.C.* 1

The electroencephalogram and cerebral ischaemia in the sheep. By B. A. BALDWIN and F. R. BELL. *Department of Physiology, Royal Veterinary College, London, N.W.* 1

Closed-circuit anaesthesia in farm animals. By S. JENNINGS. *Department of Veterinary Medicine, University of Glasgow*

The clinical assesment of cardiac output in the horse by the dye-dilution technique. By E. W. FISHER. *Department of Veterinary Medicine, University of Glasgow*

A versatile microscope for visualizing histological material with colour television. By E. C. AMOROSO and J. N. MCARTHUR. *Department of Physiology, Royal Veterinary College, London, N.W.* 1

Auditory reponses from the brain stem of pigeon. By PHYLLIS E. STOPP and I. C. WHITFIELD. *Clinical Neuropharmacology Research Center, National Institute of Mental Health, Saint Elizabeths Hospital, Washington* 20, *D.C., U.S.A.*

In pigeons anaesthetized with urethane, single unit responses have been obtained from nucleus magnocellularis, with standard micropipette recording techniques. This nucleus is considered to be the avian homologue of the ventral cochlear nucleus in mammals (Cajal, 1909).

Investigations of the range of hearing in pigeons have been made by many authors (e.g. Wundt, 1894; Wassiljew, 1933; Heise, 1953), using

variants of conditioning techniques, and have given a lower limit of about 65 c/s. Estimates of the upper limit have varied greatly ranging from 1·3 kc/s (Wundt, 1894) to as high as 12·4 kc/s (Wassiljew, 1933). More recently, values obtained by operant conditioning suggest a value of about 4 kc/s (Heise, 1953). Cochlear microphonics have been recorded over the range 50 c/s–11·5 kc/s (Wever & Bray, 1936) though the response falls markedly above 2000 c/s. In our experiments no units have been isolated responding to tones higher than 3000 c/s. At the lower end responses down to 90–100 c/s have been obtained; stimuli of lower frequency than this have not been used. Precise measurements were available only in the range 250–7000 c/s.

The threshold response curves obtained were essentially similar to those found for the cochlear nucleus in mammals (e.g. Galambos & Davis, 1943) in respect of both intensity level and shape. The majority of such curves show a characteristic frequency with the threshold rising sharply on the high-frequency side and less abruptly on the low-frequency side; however, anomalous curves of the type recently described by Katsuki, Watanabe & Suga (1959) have been seen.

As in the cat, the response of a unit to stimulation by one tone could, in many cases, be inhibited by the simultaneous presentation of a second tone, and 'spontaneous' activity could similarly be inhibited.

REFERENCES

Cajal, S. (1909). *Histologie du système nerveux de l'homme et des vertébrés.* Paris.
Galambos, R. & Davis, H. (1943). *J. Neurophysiol.* **6**, 39.
Heise, G. A. (1953). *Amer. J. Psychol.* **66**, 1.
Katsuki, Y., Watanabe, T. & Suga, N. (1959). *J. Neurophysiol.* **22**, 603.
Wassiljew, M. P. (1933). *Z. vergl. Physiol.* **19**, 424.
Wever, E. G. & Bray, C. W. (1936). *J. comp. Psychol.* **19**, 424.
Wundt, W. (1894). *Phil. Stud.* **9**, 496.

The influence of environmental temperature on the O_2 consumption of new-born rats. By P. Taylor. *Nuffield Institute for Medical Research, University of Oxford*

The O_2 consumption of an adult rat increases when the environmental temperature is reduced below the critical value (28–30° C). In the new-born rat, however, it has been concluded that O_2 consumption on exposure to cold does not increase until 1–2 weeks after birth (Antoschkina, 1939; Hahn, 1956; Adolph, 1957). Yet examination of Antoschkina's experimental results and observations by Gelineo & Gelineo (1951) and by Hahn & Koldovský (1958) have shown that, within a day or two after birth, O_2 consumption increased when the environmental temperature was

reduced from 35° to 30° C. The large number of other investigations now being carried out on the new-born rat makes it of some importance to determine the neutral temperature, at which O_2 consumption is minimal, with greater accuracy. The experiments to be described were designed with this purpose, and to determine to what extent rats can increase this O_2 consumption on exposure to cold, from birth up to 3 weeks of age.

Rats were placed in a closed-circuit respiration chamber through which air was passed and in which the rate of O_2 consumption was measured. The neutral temperature was estimated in each experiment and varied from 34° to 38° C. In new-born rats it was usually above 35° C. The minimal O_2 consumption increased from less than 20 ml./kg.min at birth to about 30 ml./kg.min at 3 weeks of age. Rats which were less than 5 hr old showed only a small increase in O_2 consumption with cooling; their stomachs contained no milk. All rats more than 5 hr old had suckled, and all but one litter showed a large increase in O_2 consumption on cooling, rising to nearly 50 ml./kg.min at 24 hr of age, and to 75 ml./kg.min or more at 2–3 weeks. The environmental temperature at which O_2 consumption was maximal fell from about 32° C at birth to less than 20° C at 3 weeks. During this period the rats increased in weight from about 5 to 30 g.

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REFERENCES

Adolph, E. F. (1957). *Quart. Rev. Biol.* **23**, 89.
Antoschkina, E. D. (1939). *J. Physiol. U.S.S.R.* **26**, 3.
Gelineo, S. & Gelineo, A. (1951). *Bull. Acad. Serb. Sci.* **3**, 119.
Hahn, P. (1956). *Nature, Lond.*, **178**, 96.
Hahn, P. & Koldovsky, O. (1958). *Nature, Lond.*, **181**, 847.

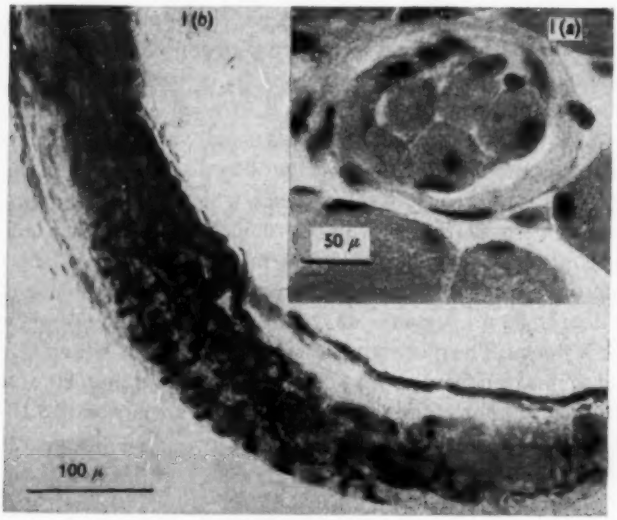
Muscle spindles in the external anal sphincter of the cat. By Mary Chennells, W. F. Floyd and R. P. Gould. *Departments of Physiology and Anatomy, Middlesex Hospital Medical School, London, W.* 1

I. PHYSIOLOGICAL FINDINGS

Action potentials were recorded from filaments of the pudendal nerve in the cat under pentobarbitone anaesthesia.

Two types of centripetal discharge were found: (*a*) impulses which came from peri-anal cutaneous endings sensitive to touch; (*b*) impulses which were increased in frequency by stretch of the external anal sphincter. The latter group of impulses were also modified by contractions of the external anal sphincter brought about by stimulation of the motor nerve and by reflex contractions of this muscle elicited through the central end of the

cut pudendal nerve. The most common modification was an increase in frequency of centripetal firing during contraction followed by a brief pause. These findings suggest the existence of a proprioceptive mechanism like the muscle spindle system of skeletal muscle.



II. HISTOLOGICAL FINDINGS

The external anal sphincter and pieces of the pudendal nerve studied in some of the physiological experiments were examined histologically.

Muscle spindles were identified: (*a*) by serially sectioning the sphincter and staining the transversely cut muscle fibres with Ehrlich's haemotoxylin and eosin (Fig. 1*a*) and (*b*) by impregnating the sphincter with gold chloride (Gairn's method) and dissecting out the individual spindles (Fig. 1*b*). An interesting feature of these spindles is that the cross-striations of the intrafusal fibres are continuous through the equatorial (nuclear bag) region.

Tissue carbohydrate reserves in foetal and new-born lambs and rhesus monkeys. By HEATHER J. SHELLEY. *Nuffield Institute for Medical Research, University of Oxford, and the National Institute of Neurological Diseases and Blindness, Field Station of Perinatal Physiology, San Juan, Puerto Rico*

The total carbohydrate concentration in tissue samples from foetal lambs and rhesus monkeys has been determined by the method of Kemp &

Kits van Heijningen (1954). Skeletal muscle carbohydrate was also determined in lambs from 3 hr to 12 days after birth and the carbohydrate content of several tissues was measured in monkeys 8–15 days after birth. Histological examination of the tissues showed that their glycogen content was proportional to their carbohydrate content. Blood glucose was determined by the method of Huggett & Nixon (1957).

Parturition occurs in sheep at about 147 days gestation age and in rhesus monkeys at about 168 days. In lambs of < 100 days gestation age, the liver carbohydrate concentration was < 15 mg/g but thereafter it increased rapidly and values of 50–100 mg/g were observed in both lambs and monkeys near term. In contrast, the lung and cardiac carbohydrate concentration decreased during the last 50 days of gestation in both lambs and monkeys, but near term there was still 3–5 mg/g in the lungs and 17–21 mg/g in the heart, 3–4 times the adult levels. The skeletal muscle carbohydrate concentration rose sharply during the last 60 days of gestation, values of > 30 mg/g being observed in lambs and monkeys near term; after birth it fell rapidly, adult values of < 15 mg/g being observed in lambs within 24 hr of birth and in all but one infant monkey. The blood glucose, which had remained below adult levels in both foetal lambs and monkeys, rose to adult levels soon after birth.

Thus foetal lambs and monkeys near term are well provided not only with liver glycogen but also with cardiac and skeletal muscle glycogen. Cardiac glycogen is depleted in anoxia and a relationship between cardiac carbohydrate and resistance to anoxia has been observed in both lambs (Dawes, Mott & Shelley, 1959) and monkeys (Dawes, Jacobson, Mott & Shelley, unpublished). The exceptionally high skeletal muscle carbohydrate concentration in these full-term foetuses is of interest in that it constitutes a carbohydrate reserve for use in the first few days after birth; the total amount of carbohydrate lost from the skeletal muscle after birth is considerable, 2–3 times the total amount present in the liver.

This work was carried out with the aid of grants from the Nuffield Foundation and the United Cerebral Palsy Research and Educational Foundation.

REFERENCES

Dawes, G. S., Mott, J. C. & Shelley, H. J. (1959). *J. Physiol.* **146**, 516–538.
Huggett, A. St G. & Nixon, D. A. (1957). *Lancet*, **273**, 368–370.
Kemp, A. & Kits van Heijningen, A. J. M. (1954). *Biochem. J.* **56**, 646–648.

The dynamic elastic modulus of the arterial wall. By D. H. BERGEL.
Department of Physiology, St Bartholomew's Hospital Medical College, London, E.C. 1

Previous measurements of arterial elasticity under static conditions predict pulse-wave velocities somewhat lower than those found experimentally (Hamilton, Remington & Dow, 1945). The arterial wall, however, is a visco-elastic material and will be stiffer when stretched rapidly; also the dilatation will lag behind the pressure.

An apparatus has been described (Bergel, 1958) with which simultaneous measurements of pressure and diameter may be made on an excised length of artery held as nearly as possible under natural conditions. The magnitudes of, and phase angle (ϕ) between, the wall tension and circumference were determined, by using small pressure oscillations about a mean of 100 mm Hg at frequencies from 2–20 c/s. The complex modulus E' may be resolved into an elastic component E dyn $= E' \cos\phi$, and a viscous component $\eta\omega = E' \sin\phi$ (Hardung, 1952; McDonald & Taylor, 1959). It was found that the latter term was always small since the phase lag only averaged 0·1–0·15 radian (5–8 degrees) and it may be neglected in estimating the dynamic elasticity.

TABLE 1. Static and dynamic elastic moduli for four types of artery at a mean pressure of 100 mm Hg. All units are dynes/cm^2 $\times 10^6 \pm$ S.E.M.

Vessel	E static	E dyn. 2 c/s	E dyn. 5 c/s
Thoracic aorta ($n = 10$)	4·4 ± 0·40	4·7 ± 0·42	4·9 ± 0·45
Abdominal aorta ($n = 7$)	9·2 ± 0·94	10·9 ± 0·88	11·0 ± 0·82
Femoral artery ($n = 5$)	9·0 ± 1·15	12 0 ± 0·81	12·0 ± 0·82
Carotid artery ($n = 6$)	6·9 ± 0·48	11·0 ± 1·00	11·3 ± 0·99

The value of E dyn was greater than the static modulus (measured 2 min after each pressure change). This increase of modulus occurred abruptly between 0 and 2 c/s and thereafter little change was found up to 20 c/s. Mean values for four different types of dog arteries are given in Table 1.

Applying these results to the prediction of pulse-wave velocity we may take the femoral artery as an example. The velocity calculated from the static modulus is 7·1 m/sec; using the dynamic modulus a value of 8·1 m/sec is obtained. This is closer to the generally accepted figure of 8–10 m/sec.

It will also be seen that the difference between the static and dynamic moduli is least in the thoracic aorta and greatest in the carotid: this seems to parallel the amount of smooth muscle in the wall as judged by histological sections.

REFERENCES

BERGEL, D. H. (1958). *J. Physiol.* **141**, 22*P*.

HAMILTON, W. F., REMINGTON, J. W. & DOW, P. (1945). *Amer. J. Physiol.* **144**, 521–535.

HARDUNG, V. (1952). *Helv. physiol. acta*, **10**, 482–498.

MCDONALD, D. A. & TAYLOR, M. G. (1959). *Progress Biophys.* **9**, 105–173, ed. BUTLER, J. A. V. & KATZ, B. London: Pergamon Press.

The effect of fleas on spleen size in voles. By D. CHITTY and ELLEN PHIPPS. *Bureau of Animal Population, Department of Zoological Field Studies, Oxford University*

When wild mammals are overcrowded in captivity they usually die or become physiologically deranged; natural populations also have high death rates when they have become crowded. It is often assumed that the experimentally induced disturbances are the same as those that occur in nature, and even Hinde (1959) fails to point out, in his meticulously fair review of certain beliefs now current among mammalogists, how much depends on this assumption and how little evidence there is for it. Indeed it is still premature to suppose that we know what disorders are commonly associated with mortality in natural populations (Chitty, 1959, 1960). Nevertheless, it seems necessary to postulate these physiological changes if we are to explain why death rates increase in certain years in nature, without any apparent increase in the severity of the external mortality factors. The gross splenic hypertrophy that occurs in wild field voles (Chitty, 1958) may be part of a syndrome induced by mutual hostility, but the field studies now in progress have not yet shown what factors initiate it. Dawson (1956) has found associated changes in haemoglobin level and reticulocyte count, and this blood condition is being studied regardless of its ultimate relevance to the population problem.

Laboratory studies, unfortunately, are complicated by many variables. The spleen enlarges in white rats and mice with certain diseases, and in voles placed among hostile strangers (Clarke, 1953; Chitty, Chitty, Leslie & Scott, 1956) or in cages containing too many fleas. Recently we have been moving animals back and forth between clean cages and those infested with fleas, which Mr M. J. Cotton has kindly identified as *Nosopsyllus fasciatus* (Bosc.), the common rat and mouse flea. Spleen weights, which are usually < 60 mg in control animals, may go up to 400 mg within 7 days of a change to a flea cage; yet animals given the same treatment, but returned to their own cages, have spleens of normal weight 5–7 days later.

Abundance of fleas is not a sufficient condition, however, for an increase in spleen size or reticulocyte count, since we frequently find a marked

response by only one of a pair of voles occupying the same cage; and litter mates treated alike may respond differently, as may animals newly brought in from the field. Some of these variations presumably reflect individual differences in physiological condition, and if our present tests are successful we may eventually be able to use them to show whether changes in susceptibility really do occur in nature, an assumption we have hitherto found no practicable means of verifying.

REFERENCES

Chitty, D. (1958). *Cold Spr. Harb. Symp. quant. Biol.* **22**, 277–280.
Chitty, D. (1959). *Ecology*, **40**, 728–731.
Chitty, D. (1960). *Canad. J. Zool.* **38** (in the Press).
Chitty, D., Chitty, H., Leslie, P. H. & Scott, J. C. (1956). *J. Path. Bact.* **72**, 459–470.
Clarke, J. R. (1953). *J. Endocrin.* **9**, 114–126.
Dawson, J. (1956). *Nature, Lond.*, **178**, 1183–1184.
Hinde, R. A. (1959). In *The Nature of Stress Disorder*, pp. 94–101, 123–126. London: Hutchinson Medical Publications Ltd.

Alterations in the fusion frequency of flicker correlated with electroencephalogram changes at increased partial pressures of nitrogen. By P. B. Bennett and A. V. C. Cross. *Royal Naval Physiological Laboratory, Alverstoke, Hants*

When men are exposed to raised pressures of air, changes in brain-wave activity occur (Bennett & Glass, 1957*a*, *b*). The alpha-blocking response, in which the 8–13 c/s alpha activity blocks to mental stimulation, is abolished. The time from the start of compression, until the abolition of blocking, was found to be inversely proportional to the square of the pressure. It was possible only to record this change in 'Responsive' subjects, comprising about 47 % of any group. Non-responsive subjects do not show alpha blocking.

The fusion frequency of flicker has been tried as an alternative. A flicker fusion meter, as described by Fritze & Simonson (1951), was used, the rate of flicker being controlled electronically from 20 up to 100 c/s with a small neon lamp as a light source. A subject sat in the dark, so that he faced the window of the recompression chamber. The neon lamp, mounted in a black wood surround, was outside the chamber but against the window. The frequency of flicker was slowly increased until the subject indicated that the flicker was no longer perceptible. Three readings were taken at each recording on the surface and approximately every minute at pressure. The electroencephalograms of 'Responsive' individuals were also recorded and the time to abolition of the alpha-blocking response or 'nitrogen threshold' noted.

At pressure there is a maintained alteration of the fusion frequency of flicker. The majority of subjects show a fall and a few a rise of one or more flashes per second. The time to this change is inversely proportional to the square of the pressure, i.e. $P\sqrt{T}$ is a constant. The value of this constant depends upon the individual. Agreement with this relationship is striking. Such agreement suggests a biophysical action for the nitrogen in the production of the 'nitrogen threshold'.

The experiments using fusion frequency of flicker and the electroencephalogram show that the times to produce alpha-blocking abolition and the maintained alteration of fusion frequency of flicker are the same and support is obtained for the theory first postulated on the basis of electroencephalogram results (Bennett & Glass, 1957*a*, *b*) that a fundamental change occurs in the central nervous system when a certain tension of nitrogen or other inert gas is reached.

It is hoped that measurement of the reaction of men to nitrogen or other physiologically inert gases may be of value in the selection of divers and in answering some of the more fundamental problems on the effects of nitrogen at increased pressures.

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REFERENCES

Bennett, P. B. & Glass, A. (1957*a*). *J. Physiol.* **138**, 18–19*P*.
Bennett, P. B. & Glass, A. (1957*b*). *Admiralty Report. U.P.S.* **170**, R.N.P.L. 11/57.
Fritze, C. & Simonson, E. (1951). *Science*, **113**, 547.

The effect of suggestion under hypnosis on the circulatory response to thermal stimuli. By S. Black, O. G. Edholm, R. H. Fox and D. J. Kidd. *Division of Human Physiology, National Institute for Medical Research, London, N.W. 3*

Subjects who can be hypnotized into a deep trance have been examined in a variety of thermal states. Blood flow through the hand and forearm, respiratory and heart rates, have been measured with the subjects reclining in a water-bath maintained at 34° C. Induction of hypnosis produced a variable response, but on the average hand blood flow was reduced and forearm blood flow was not changed. Experiments performed have included: (*a*) suggestions of heat or cold, both general or confined to a hand or forearm, without any actual change in temperature; (*b*) suggestions of heat or cold when the bath temperature was being changed in an opposite direction. Observations have also included the effect of suggestion on sweating.

Suggestion of general body heating does not alter body temperature, nor has it proved possible to increase hand or forearm blood flow so as to mimic the usual response to general body heating. Similarly, suggestion does not inhibit the circulatory response to general body heating.

The circulatory responses to thermal suggestion vary considerably from subject to subject and also in the same subject. In certain cases the response to suggestion by the hypnotist closely resembled the circulatory changes observed with emotional stimuli. Suggestion of general body heating was followed by a small increase in blood flow in seven out of sixteen observations on the hand, and in nine out of twenty-five observations on the forearm. But in a number of cases there was a fall in blood flow and in the remainder no change was seen. When suggestions of cold were made, blood flow never increased, and a small fall was observed in about half the experiments. It is concluded that suggestion under hypnosis does not significantly modify the normal circulatory response to thermal stimuli.

The activity of the cold vasodilatation phenomenon in various body surface areas of man. By R. H. Fox and H. T. Wyatt. *Division of Human Physiology, National Institute for Medical Research, London, N.W. 3*

Evidence for vasodilatation on exposing the fingers and toes, the ear and some other parts of the face to cold was described by Lewis (1930), who also noted that it could only rarely be obtained from the forearm. Cold vasodilatation in the forearm has also been reported by Clarke, Hellon & Lind (1957).

In the present study, thirty-three sites on the body surface have been tested using a calorimeter designed to cool a small area of skin. The subjects were two adult males; one 30 min experiment was performed at each site on both subjects. To ensure release of vasoconstrictor tone, the subjects always exercised until the first appearance of sweat on the forehead, and were examined reclining in a warm room wearing additional clothing.

The results have been divided into three groups (Table 1): Group I—Negative. The heat loss curves took an exponential form. Group II—Equivocal or weakly positive results. Deviation from the simple exponential curve but no rise in heat loss greater than 50 kcal/m^2/hr. Group III—Positive. A rise in heat loss exceeding 50 kcal/m^2/hr. For the two sites on the back of the hand and the one on the forearm, included in Group II, one subject was positive and the other negative.

With more experiments and other subjects, or a stronger stimulus, a positive response might be elicited from areas so far doubtful or negative,

TABLE 1. Classification of sites

Area	Group III	Group II	Group I
Head and neck	Forehead; ala of nose; cheek; lip; submandibular; pinna of ear; mastoid		Vertex of skull
Thorax and abdomen	Perianal; buttock	Head clavicle; medial to nipple; lat. to umbilicus; umbilicus	Nipple; back 3 in. (7·5 cm) lat. to L1; scrotum
Arm and hand	Olecranon; palm	Mid flexor forearm; lat. dorsum hand; mid dorsum hand; mid extensor forearm	Mid arm lat.
Leg and foot	Patella; heel; pad great toe; mid sole laterally	Mid calf; mid dorsum of foot	Mid anterior thigh; popliteal fossa; mid sole medially

but it is suggested that the pattern found in these experiments shows the areas in which the cold vasodilatation phenomenon is most active.

REFERENCES

CLARKE, R. S. J., HELLON, R. F. & LIND, A. R. (1957). *J. Physiol.* **137**, 84*P*.
LEWIS, T. (1930). *Heart*, **15**, 177.

The effect of tilting from the horizontal to the head upwards position on the pressure of the cerebrospinal fluid in the lumbar subarachnoid space of the dog. By T. H. B. BEDFORD. *Department of Pharmacology, University of Manchester*

Evidence has been supplied in an earlier communication (Bedford, 1958) that tilting to the head upwards position is unaccompanied by any marked change in the pressure of the cerebrospinal fluid in the cisterna magna. In the present series of experiments a study has been made on seven dogs of the effect of tilting through an angle of 45° from the horizontal to the head upwards position on the pressure of the cerebrospinal fluid in the lumbar subarachnoid space. The general experimental technique was that used in earlier experiments. The lumbar subarachnoid pressure was measured with the needle introduced between the 3rd and 4th lumbar vertebral spines. It was found essential to obtain clear cerebrospinal fluid on first puncture. Inconsistent results could nearly always be traced to faulty puncture. Experiments in which the cerebrospinal fluid contained blood were disregarded. The intracisternal pressure was recorded throughout the experiments in the usual way. The mean arterial blood pressure was recorded in the femoral artery.

In the horizontal position with the head slightly raised and supported in as natural position as possible, the lumbar subarachnoid pressure when measured from the point of insertion of the needle through the skin was generally found to be somewhat higher than the cisternal pressure recorded in the same way. The tops of the columns of cerebrospinal fluid in the two manometers were, however, invariably in the same horizontal plane. On tilting through an angle of 45°, to the head upwards position, the pressure of the cerebrospinal fluid in the cisterna magna displayed changes corresponding to those previously reported and finally reached a level 10–30 mm higher than the original one. Thereafter the pressure, apart from minor variations, remained stationary. The lumbar subarachnoid pressure rose rapidly without any initial fall until the column of fluid in the recording manometer had reached a level in the same horizontal plane as that in the cisternal manometer. This was attained 2–7 sec after tilting. The pressure then remained stationary or displayed variations corresponding to those observed in the cisterna magna. On returning the animals to the horizontal position, the original levels were resumed. Artificial variations in the intracisternal pressure with the animals in the horizontal or the tilted position were at once reflected in the lumbar pressure and vice versa. The new levels were invariably in the same horizontal plane.

It would appear from these experiments that the lumbar subarachnoid space remains in free communication with the cisterna magna during tilting to the head upwards position and that the magnitude of the rise in the lumbar subarachnoid pressure is determined by the necessity to maintain the intracranial subarachnoid pressure within a fixed range. Although the precise nature of the mechanism involved awaits elucidation, it is probably of vascular origin.

REFERENCE

BEDFORD, T. H. B. (1958). *J. Physiol.* **145**, 9–10*P*.

Arterial air embolism during decompression under water, and its prevention. By M. S. MALHOTRA and H. C. WRIGHT. *Royal Naval Physiological Laboratory, Alverstoke, Hants*

The incidence of arterial air embolism during diving and in submarine escape training is not very common; nevertheless it is viewed with considerable concern. Investigations have been made into causes and methods of prevention with rabbits. Simulated ascents have been made in a compression chamber at the rate of 5 ft. (152 cm) sec, from equivalent depths of 100 ft. (30·5 m) of sea water. Three series of experiments have

been done. In all these, the animals were tracheotomized and the outlet from the trachea closed at 60 ft. (18·3 m).

In the first, or control series, the peak intratracheal pressure ranged from 60 to 90 mm of mercury, and all the animals died. In the second series, the chest was squeezed manually just before the trachea was closed and all the animals survived, the pressure in the chest rising only to 10–18 mm Hg. In the third series, a firm binder was applied to the lower abdomen; all the animals survived without any evidence of injury, and the intratracheal pressures ranged from 24 to 50 mm of mercury.

The main injuries observed in the first series were interstitial emphysema, air embolism, pneumothorax and haemothorax. The quantity of air in the circulation was noticeably greater when interstitial emphysema was marked and was very much less when pneumothorax was present. Air appeared in the circulation only after the intrapulmonary pressure was allowed to fall to atmospheric. In none of these animals was lobular expansion of the lungs seen. There was, however, a generalized injury to the distensible parts of the lungs.

Manual squeezing of the chest before clamping the trachea or binding of the lower abdomen have both been found to provide protection against air embolism. We have concluded that squeezing the chest acts by reducing the volume of air in the lungs nearly to 'residual', and that during decompression this relatively small amount of air can expand without producing over-distension and damage. Further work will show whether this would provide protection over a greater pressure range. Presumably the protection offered by the abdominal binder is brought about by raising the diaphragm, which not only provides a support to the under surface of the lungs but also reduces the initial lung volume at the start of the ascent.

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Changes in cell density along the growth axis of the pulp of the continuously growing rabbit mandibular incisor. By EILEEN A. T. GAGAN and A. R. NESS. *Medical Research Council, Physiology Department, University College London*

Counts have been made, on composite photomicrographs at enlargements of about × 100, of cell nuclei in the pulps of rabbit mandibular incisors; sections being cut along the growth axis, 4 μ thick and stained with haematoxylin. The odontoblast layer was excluded from the counts but cells lining the pulpal sinusoids were included. The constant cross-section of the adult incisor allows the reconstruction of the true outline of a pulp: thus

estimates were made of area (excluding the lumens of pulpal sinusoids) and cell densities were computed (Fig. 1).

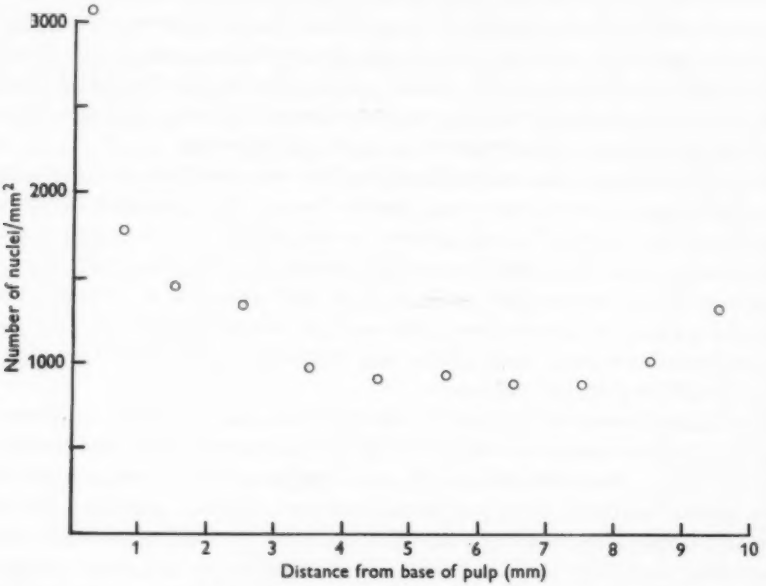

Fig. 1. Distribution of cell nuclei in the pulp of the rabbit incisor tooth. Each point represents the mean of counts from photomicrographs of one section from each of the two mandibular incisors of four rabbits, and is plotted in the middle of the region over which the count was made.

There is good evidence that the pulp grows, continuously, away from the tooth base: if so, then the inverse process of tissue removal must operate, since pulpal volume decreases as dentine is deposited, eventually to obliterate the pulp chamber. It is suggested that the fall in density of nuclei (Fig. 1), rapid in the basal 2 mm, is due to cells being forced apart by tissue fluid extruded from the blood vessels. Presumably some of the tissue fluid constituents would then be organized into the solid phase—the result being interstitial growth of pulpal tissue. The resultant tendency for cell density to fall would be balanced by cell proliferation, which takes place mainly at the periphery of the pulpal base (Ness & Smale, 1959), with migration of cells into the region of interstitial growth. As the pulp diminishes in cross-section so cells and extracellular material must be destroyed and removed. It is thought that this process becomes dominant over proliferation at about 5 mm from the pulpal base; the rise in cell

density, starting at about 8 mm from the base (Fig. 1), may result from extracellular material being removed faster than cellular.

REFERENCE

NESS, A. R. & SMALE, D. E. (1959). *Proc. Roy. Soc.* B, **151**, 106.

Heart sounds in the Knowlton–Starling heart–lung preparation of the dog. By A. W. SLOAN, *Physiology Department, University of Cape Town Medical School, Observatory, Cape Town, South Africa*

The identification of acetylcholine, 5-hydroxytryptamine and other substances in hornet venom (*Vespa crabro*). By K. D. BHOOLA,* J. D. CALLE and M. SCHACHTER. *The Physiology Department, University College London*

Acetylcholine (ACh) was present in concentrations varying from 18 to 50 mg/g dry venom sac (mean, 32·6) in tests on six sacs. Identification was by parallel assay on the isolated guinea-pig ileum and frog rectus abdominis muscle. These actions were antagonized by atropine and by tubocurarine respectively. Also, the active substance was eluted from paper chromatograms of ethanol (95%) extracts of the venom sacs and its R_F values in several solvents (*n*-propanol/formic acid/water, 8:1:1; *n*-butanol/acetic acid/water, 4:1:5) were identical with those of acetylcholine. The active area, like acetylcholine, developed with iodine.

5-Hydroxytryptamine (5-HT) was separated from other active substances in the venom by paper chromatography, and eluted and identified. It was identical with 5-HT in its actions on the rat uterus and guinea-pig ileum and was antagonized by lysergic acid diethylamide (LSD). Its identity was confirmed by paper chromatography in six solvent systems. The concentrations of 5-HT in the venom were determined by bioassay of chromatogram eluates. The concentrations in three separate groups of venom sacs were 7·5, 14·7 and 19·0 mg/g dry venom sac.

Histamine was present in concentrations varying from 14 to 30 mg/g dry venom sac (mean, 19·8). The concentration was always less than that of ACh (approximately one half) in the same venom sac. Identification with histamine was made by its action on the guinea-pig ileum, its antagonism by mepyramine, and by paper chromatography.

A new kinin was found both in the ethanol (95%) extract and in the ethanol insoluble residue of the venom sac. This substance was closely

* M.R.C. Scholar.

associated with 5-HT on chromatograms of the ethanol extract in *n*-propanol/formic acid or in *n*-butanol/acetic acid solvents. The activity of the eluate with the R_F values and colour reactions of 5-HT was only partially abolished by LSD on the rat uterus, but the remaining activity due to the kinin was abolished by incubation with chymotrypsin (20 μg/ml., 10 min, 35° C), though unaffected by incubation with trypsin. The kinin was completely separated from 5-HT, however, by rechromatographing the eluate in ethanol/0·2N hydrochloric acid (1:1). Pharmacological and paper chromatography experiments indicated that the kinin in the ethanol extract of the venom was identical with the kinin in the ethanol insoluble residue. In parallel tests on the rat uterus, rat duodenum, guinea-pig ileum and rabbit arterial blood pressure, it was readily distinguished from oxytocin by its relatively greater effectiveness in relaxing the rat duodenum (Gaddum & Horton, 1959) and by its potent hypotensive activity. It resembled bradykinin closely in all the tests except that it was approximately 8 times less active on the guinea-pig ileum.

The concentrations of ACh and 5-HT are the highest yet reported. The histamine concentrations are comparable to those in wasp and bee venom (Schachter & Thain, 1954), but the kinin in wasp venom (Holdstock, Mathias & Schachter, 1957) was not detected in the hornet.

REFERENCES

Gaddum, J. H. & Horton, E. W. (1959). *Brit. J. Pharmacol.* **14**, 117.
Holdstock, D. J., Mathias, A. P. & Schachter, M. (1957). *Brit. J. Pharmacol.* **12**, 149.
Schachter, M. & Thain, E. M. (1954). *Brit. J. Pharmacol.* **9**, 352.

Daily changes in the buoyancy of the cuttlefish. By E. J. Denton and J. B. Gilpin-Brown. *Marine Biological Association of the United Kingdom, Plymouth*

By pumping liquid in or out of the rigid cuttlebone so as to change the volume of the gas spaces which it contains, the cuttlefish (*Sepia officinalis* (L)) can make itself more or less dense than sea water (Denton & Gilpin-Brown, 1959). The following experiments give an example of the use of this mechanism during the life of the animal.

The behaviour of a well-fed cuttlefish is strikingly affected by light. When the light is bright they usually bury themselves in the gravel at the bottom of their tanks, whilst after twilight they come out of the gravel and swim around until dawn. Now a cuttlefish can be gently shepherded into a box without being much disturbed and its density found by weighing it *under sea water* in a water-tight box filled with sea water. If, for example, an animal weighs minus 3 g in sea water then the weight of the box + animal + sea water will be 3 g less than the weight of the box filled with

sea water only. Animals kept in artificial light and dark showed quick changes in density which could amount to over 1 %. These changes could only come from a change in the volume of the gas space within the cuttlefish. There was no trace of gas within the mantle cavities and at the end of the experiment the densities of the cuttlebones were found to be those predicted on the hypothesis that the cuttlebone was the site of the density changes.

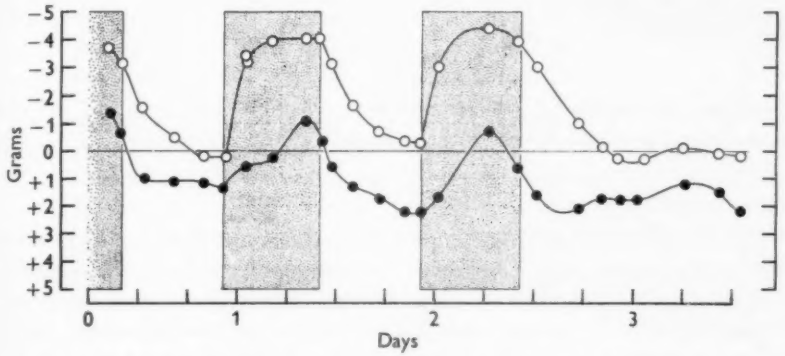

Fig. 1. *Sepia officinalis*. Changes of weight in sea water of two specimens. The upper curve is for an animal weighing around 330 g, the lower curve for an animal weighing around 260 g. The ordinate shows the weight of an animal in sea water (negative weights mean that the animal was less dense than sea water). The dark areas indicate the times of darkness.

These experiments show that the cuttlefish is able to change its density at quite rapid rates, and does so to help it to lie at the bottom during the day and to keep off the bottom during the night.

REFERENCE

DENTON, E. J. & GILPIN-BROWN, J. B. (1959). The buoyancy of the cuttlefish. *Nature, Lond.*, **184**, 1330–1331.

The removal of bromsulphthalein from the plasma in man.

By G. BARBER-RILEY, A. E. GOETZEE, T. G. RICHARDS and J. Y. THOMSON. *Physiological Laboratory and the Department of Applied Mathematics, University of Liverpool*

It has been shown (Richards, Tindall & Young, 1957) that in the dog the disappearance of bromsulphthalein from the plasma following a single I.V. injection has a form which, in general, may be expressed by

$$C_{(t)} = Ae^{-k_1 t} + Be^{-k_2 t},$$

where $C_{(t)}$ is the plasma concentration at a time (t) from the injection, and A, B, k_1 and k_2 are constants. The simplest explanation is that of three 'compartments', where dye passes from the plasma to the liver and from the liver to the bile. It is a system which, in regard to dye, never runs to equilibrium since the last 'compartment' is of infinite size, i.e. the bile is lost.

The conditions above are fundamentally different from those in which two 'compartments' of finite size run to a simple equilibrium, in which case the equation

$$C_{(t)} = A\,e^{-kt} + B$$

describes the decline of concentration in the first compartment. If the second compartment is very large indeed, the above becomes

$$C_{(t)} = A\,e^{-kt}.$$

It has been reported (Inglefinger, Bradley, Mendeloff & Kramer, 1948) that in the human subject bromsulphthalein loss from plasma is normally of this 'single exponential' (2-compartment) form. However, bromsulphthalein injected I.V. in man certainly appears in the bile, and this appears to be preceded by passage through the liver, and is thus qualitatively similar to the dog. The plasma curve should therefore be of a '3-compartment' form unless (*a*) the second 'compartment' were so large (and the second term so small) that no visible evidence of it is seen at measurable plasma levels, or (*b*) the singular condition exists that k_1 is virtually equal to k_2 (so that A and B become separately indeterminate).

We have obtained fifty-five bromsulphthalein disappearance curves in forty-four normal fasting volunteers. The usual standard I.V. dose of bromsulphthalein was 200 mg/m^2; some of the subjects received 150 mg/m^2. It was usual to alter the dosage in repeated tests in the same subject. Approximately fourteen blood samples were taken over a period of 60–70 min. An electronic computer was used in the analysis of the results in each test. In all but seven of these a 'two exponential' form resulted, i.e. qualitatively but not quantitatively similar to the dog. We believe it to be unlikely that 'saturation' could explain these results, and it was observed that reduction of dosage in the same subject produced no significant change in the dye transfer rates; 'transfer rates' in this context means the proportional loss of dye from the plasma or the liver in unit time. Quantitatively our experiments suggest that the transfer of dye from the plasma to the liver is twice as great in the dog as it is in man, but that transfer of dye from the liver to the bile is about three times as great in man as it is in the dog.

REFERENCES

INGLEFINGER, F. J., BRADLEY, S. E., MENDELOFF, A. I. & KRAMER, P. (1948). *Gastroenterology*, **11**, 646–657.

RICHARDS, T. G., TINDALL, V. R. & YOUNG, A. (1957). *J. Physiol.* **138**, 38*P*.

Effect of clamping carotid and vertebral arteries on blood pressure in the sheep. By B. A. BALDWIN and F. R. BELL. *Department of Physiology, Royal Veterinary College, London, N.W.* 1

Pressure changes produced locally and systemically by clamping the carotid and vertebral arteries have been investigated in sheep anaesthetized with thiopentone-sodium. Pressures were recorded by mercury manometers from both lingual arteries and a femoral artery. Respiratory movements were also recorded. Arteries exposed for clamping were both common carotids in the mid-neck region, the 'occipital group' comprising the occipital and ascending pharyngeal arteries, the external carotids and both vertebrals as they entered the transverse canal of the 6th cervical vertebra.

Clamping one common carotid artery produces a fall of 22–40 % of the initial pressure in the ipsilateral lingual artery; there is no pressure change in the contralateral lingual or systemic pressures. Clamping both carotids produces a fall of 45–70 % in the pressure of both lingual arteries, the systemic pressure showing a small reflex rise. Clamping one or both vertebral arteries has no effect on lingual pressure or on systemic pressure. Clamping both common carotids together with both vertebrals produces a pressure fall of 87–90 % in both linguals, and a marked rise in systemic pressure and apnoea. Restoration of the systemic pressure to its normal level occurs rapidly on release of either the carotid or vertebral arteries. A similar systemic hypertension and apnoea can be produced by clamping both common carotids together with both occipital arteries. Release of the occipitals, which allows vertebral blood to supply the whole head, relieves the systemic pressure and apnoea. Clamping both external carotid arteries only also produces apnoea and systemic hypertension but in this case the rise is reflected in the lingual arteries, indicating that the systemic hypertension is due to central ischaemia since the sinus region is well supplied with blood at high pressure.

The experiments show that communication between the vertebral arteries and the ventral spinal and basilar arteries is inadequate to maintain a normal cerebral blood supply. They provide further evidence for the observation of Linzell & Waites (1957) that vertebral blood can

maintain cerebral function in the sheep when both carotids are clamped by entering the external carotid arteries via the occipital arteries.

This work was supported by a grant from the Animal Health Trust.

REFERENCE

LINZELL, J. L. & WAITES, G. M. H. (1957). *J. Physiol.* **138**, 20*P*.

The origin and course of motor nerve fibres to the oesophagus.

By A. M. LAWN. *Department of Physiology, Royal Veterinary College, London, N.W.* 1

Brief electrical stimuli were applied to the medulla oblongata of rabbits, deeply anaesthetized with urethane, by means of a fine wire electrode (insulated except at the tip) manipulated by a stereotaxic instrument. Oesophageal activity was detected by leading off the oesophageal electromyograph through miniature, bipolar, suction electrodes applied to the surface of the oesophagus in the neck and near the cardia.

At each locus of stimulation stimuli of five different strengths were applied while the oesophageal electromyograph was recorded by amplifiers and an oscilloscope. The loci were spaced 0·5 mm from each other vertically and horizontally. In addition, at each insertion point the loci of lowest threshold were found, marked by electrolytic deposition of iron from the electrode and subsequently identified by the Prussian blue reaction in serial frozen sections 100 μ thick.

Loci giving low-threshold responses were found throughout a plane extending from immediately ventral to the dorsal motor nucleus of the vagus nerve to the origin of the vagus rootlets laterally. The most rostral loci lay over the rostral part of the facial nucleus and the most caudal loci lay 1 mm caudal to the level at which the facial nucleus gives place to the nucleus ambiguus. This horizontal plane coincided with the position of the fibre bundles entering and leaving the vagal rootlets. In addition deeper loci giving low-threshold responses were found extending from immediately ventral to the dorsal motor nucleus to a mass of cells forming the enlarged rostral extremity of the nucleus ambiguus (ventral motor nucleus of the vagus nerve). The occurrence of high-frequency 'injury' type discharges in the oesophagus only after the stimulating electrode had been advanced into this cell mass is evidence that the cell bodies of the oesophageal motor neurones lay in this region.

The results of these experiments suggest, therefore, that the motor neurones innervating the oesophagus are situated in the rostral part of the nucleus ambiguus and that their axons follow, first a dorsomedial then a lateral course to reach the vagal rootlets.

By this technique loci of three types were identified, stimulation of which at low intensity gave rise respectively to a response in the cervical oesophagus only, a response in the abdominal oesophagus only, or to responses in both regions of the oesophagus.

The loci of the cervical type were not more rostrally distributed than those of the abdominal type; instead, all the loci appeared to be distributed as a single group.

The cremaster muscle and testicular descent in the rat. By K. M. Backhouse*. *Charing Cross Hospital Medical School, London, W.C.* 2

A so-called inverted cremaster muscle has been invoked, with muscular fibres of the gubernaculum testis, as the propulsive force in testicular descent. Lewis (1948) gave support to this view by cutting the genitofemoral nerve supply to the muscle in rats under 10 days of age, thus preventing testicular descent of puberty. The gubernaculum testis, however, has now been shown to be a mesenchymatous structure without active contractile components. In the rat, at birth, the cremaster sac is already formed and contains the descended testis and cauda epididymis. But complete histological differentiation of the mesenchymatous gubernacular structures to fibrous tissue and of the cremaster muscle is not completed until several days after birth. The so-called testicular descent of puberty consists of hypertrophy of the genital system including the containing cremaster and scrotal sacs, a different process from foetal testicular descent.

Thirty rats were divided into six equal groups. The genitofemoral nerve was cut unilaterally at successive 4-day intervals in each group, starting on the fourth post-natal day. Controls were operated on without cutting the nerve. In the 4- and 8-day groups the cremaster muscle on the ipsilateral side failed to develop further and to hypertrophy at puberty. Animals so treated from 12 days onwards behaved normally at puberty: the cremaster muscle, although inactive, hypertrophied to contain the enlarged testis and epididymis as did all the controls.

Selye (1943) showed that removal of the testis on one side prevents pubertal hypertrophy of the scrotum on that side unless an artificial testis mass is substituted. This experiment was repeated in twelve rats denervated at 12 days, a glass ball of comparable size being sewn into the tunica albuginea of the affected side in place of the testis. In each case the scrotal sac expanded normally at puberty. In five other denervated rats aged 12 days, the glass ball fitted was too large to slide into the pelvic mouth of

* Experimental work carried out in the University of Florida.

the scrotal sac. In these cases there was no hypertrophy of the cremasteric sac on that side. Removal of both testes and replacement with glass balls invariably lead to no cremasteric hypertrophy at puberty.

It appears therefore that an intact nerve supply is essential for the cremaster muscle until it has completed its growth processes at about 10 days of age. Thereafter, if the hormonal environment is correct, and the cremasteric sac contains sufficient bulk, pubertal hypertrophy will take place even in a denervated cremaster muscle, though the testis will not be retractible on that side.

REFERENCES

LEWIS, L. G. (1948). *J. Urol.* **60**, 345–356.
SELYE, H. (1943). *Anat. Rec.* **85**, 377–386.

The effect of sodium depletion on the taste threshold of calves.

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The value of monovular calves for the study of physiological sensory mechanisms has been demonstrated by Bell & Williams (1959) in an investigation of discrimination thresholds for a variety of sapid solutions, including NaCl.

Denton (1957) has described a method of inducing sodium deficiency in sheep by exteriorizing a parotid duct when the continuous secretion of saliva causes a loss of sodium from the body. Sodium-depleted sheep show an avidity for rock salt, the intake increasing from 0·5–2·0 g/day in the normal state to 5–15 g/day when depleted. Sodium depletion in sheep with a parotid fistula can be prevented by feeding a daily supplement of 50 g $NaHCO_3$.

The parotid duct of one member of a pair of mono-zygotic cattle twins was exteriorized with effects very similar to those described for sheep. The salivary Na:K ratio falls progressively from 145 m-equiv/l.: 4·5 m-equiv/l. to 5·4 m-equiv/l.: 132 m-equiv/l., but it can be restored to normal on feeding supplements of NaCl or $NaHCO_3$. Salivary output from the exteriorized parotid duct decreases with the degree of sodium depletion. A sodium-depleted calf is able to self-select salt solution from a variety of other sapid solutions. When allowed continuous access to either 1% NaCl or 2% $NaHCO_3$ it will take sufficient to maintain its salivary Na/K ratio at normal levels.

After a period of depletion the taste thresholds for both members of the twin pair have been examined, by the discrimination technique (Bell &

Williams, 1959). The calf with the exteriorized parotid duct shows a higher threshold for acceptance of sodium chloride for it will take 5 % NaCl while the normal calf will accept only 0·03 % NaCl. In tests for $NaHCO_3$ taste thresholds the experimentally sodium-depleted calf showed a higher acceptance threshold (8 %) than the normal calf (0·25 %).

During the discrimination tests the salivary Na/K ratio was restored to normal in the experimental calf when it was taking the higher concentrations of either NaCl or $NaHCO_3$. With the more dilute solutions, however, the animal did not take sufficient of either salt to maintain a normal salivary Na/K ratio. At first, during the test, with decreasing dilution the amount of test solution imbibed was also increased as though the animal was responding by some internal regulatory mechanism of the kind postulated by Richter (1942–3), but with the lower dilutions the calf failed to increase its intake further and consequently became sodium depleted. This limitation was not due to water satiety, for the control twin ingested a similar total fluid volume.

REFERENCES

Bell, F. R. & Williams, H. L. (1959). *Nature, Lond.*, **183**, 345.
Denton, D. A. (1957). *Quart. J. exp. Physiol.* **42**, 72.
Richter, C. P. (1942–3). *Harvey Lect.* **38**, 63.

Local activation of slow fibres from striated muscle of the frog. By A. F. Huxley and L. D. Peachey. *Physiological Laboratory, University of Cambridge*

Temperature control in chicks of the Adelie penguin. By R. Goldsmith and W. J. L. Sladen. *Department of Pathology, Johns Hopkins Hospital, Baltimore, U.S.A. and Department of Physiology, Medical Research Council Laboratories, Holly Hill, London, N.W.* 3

The part played by aldosterone in the urinary changes of standing. By J. N. Mills, S. Thomas and K. S. Williamson. *Department of Physiology, University of Manchester*

We have shown (Gowenlock, Mills & Thomas, 1959) that aldosterone excretion rises during 3 hr of standing, and have suggested that some of the urinary changes on standing up are due to increased production of aldosterone. A fuller examination of the part played by adrenal steroids has been made possible by the use of the spirolactone SC-8109 (Searle),

which has no direct action on the kidney but blocks the action of aldosterone, and probably other steroids, upon renal kation excretion.

We ourselves were the subjects of the experiments. First, it was shown that SC-8109 (500 mg intramuscularly, in 5 ml. 20% benzyl alcohol, 50% propylene glycol, 30% water) was without effect on the recumbent subject but blocked the effect of intravenous aldosterone during 4 hr of observation. Renal electrolyte outputs were then examined in subjects standing from 13.00 to 17.00 hr with and without SC-8109. The difference between these two experimental situations represents the effect of endogenous aldosterone upon the standing subject, which may be compared with the effect of exogenous aldosterone upon the recumbent subject at the same time of day. This was given as 500 μg of the D-isomer, in one or two doses, or as a continuous drip at 38–53 μg/hr for 3 or 4 hr. Comparison between recumbent subjects, in whom aldosterone production is believed to be minimal, and subjects standing with SC-8109, will show the renal effects of postural change by all mechanisms other than altered aldosterone (or other mineralocorticoid) production. This non-adrenal effect of standing, referred to for convenience as the 'haemodynamic' effect, was to reduce the excretion of Na and K, and the Na : K ratio. Aldosterone, endogenous or exogenous, roughly halved the Na excretion; the action was more striking when exerted on the high Na output of the recumbent, than on the low Na output of the standing, subject. Aldosterone elevated the potassium output of the standing or recumbent subject, and thus lowered the urinary Na : K ratio to much the same extent as did standing with steroid effects blocked.

It is concluded that standing up decreases the excretion of Na by two methods, increased aldosterone production, and a supposed haemodynamic change, and that the two effects are additive. Potassium excretion is increased by aldosterone and reduced by the haemodynamic change, and the two effects largely cancel one another.

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REFERENCE

GOWENLOCK, A. H., MILLS, J. N. & THOMAS, S. (1959). *J. Physiol.* **146**, 133–141.

Antidiuretic activity and hyaluronidase in human urine. By S. E. DICKER and M. GRACE EGGLETON. *Departments of Pharmacology and Physiology, University College London*

The medullary tissue of the mammalian kidney appears to be hypertonic to plasma, increasing in tonicity from the cortical boundary to the papilla. Moreover, the tubular fluid in the first half of the distal tubule is hypotonic

to plasma and emerges unchanged into the renal pelvis in the absence of ADH, as in water diuresis. In its presence, the fluid becomes isosmotic in the latter half of the distal tubule and is hypertonic at its exit from the collecting tubule. These facts can be accounted for by supposing that the hormone makes the relevant sites more permeable to water, simple osmotic forces then accounting for the resultant changes in tonicity of the fluid. Ginetzinsky (1958) found hyaluronidase in the urine of dehydrated animals and noted that it disappeared during water diuresis. He produced histological evidence of depolymerization of hyaluronic acid in the intercellular matrix of the collecting duct epithelium during dehydration and suggested that ADH stimulated liberation of hyaluronidase by the epithelial cells in this region, in this way making the tubule wall more permeable. The concentration of ADH itself in the urine reflects that in the blood plasma. It seemed of interest, therefore, to see whether excretion of hyaluronidase could be correlated with that of ADH and generally to attempt to confirm Ginetzinsky's results.

Hyaluronidase (1–10 u./ml.) was present in all urine samples tested at basal rates of urine flow, as was ADH (100–22,000 μu./ml.), but there was no quantitative relation between the two when different individuals were compared or even in any one individual on different occasions. During the height of a water diuresis, however, both ADH and hyaluronidase concentration in the urine were greatly diminished and the absolute amount excreted decreased in the four subjects examined. Ingestion of alcohol (four subjects) produced identical effects. These results in man, therefore, agree with Ginetzinsky's findings in animals, but the lack of any quantitative relation between the amounts of ADH and hyaluronidase excreted suggests the presence of many variable factors in the mechanism of ADH action.

In the new-born infant (0–2 days), urine is slightly hypertonic to plasma but is not further concentrated by exogenous ADH, in contrast with older infants whose urine is hypotonic and who show a small response to ADH (Heller, 1944). The first urine passed by eight new-born infants was, therefore, examined for the presence of ADH and hyaluronidase. It contained ADH 25–900 μu./ml. and hyaluronidase 0·7–5·2 u./ml. The combined findings suggest that the medulla of the infant's kidney may be less hypertonic than that of the adult.

REFERENCES

GINETZINSKY, A. G. (1958). *Nature, Lond.*, **182**, 1218–1219.
HELLER, H. (1944). *J. Physiol.* **102**, 429–440.

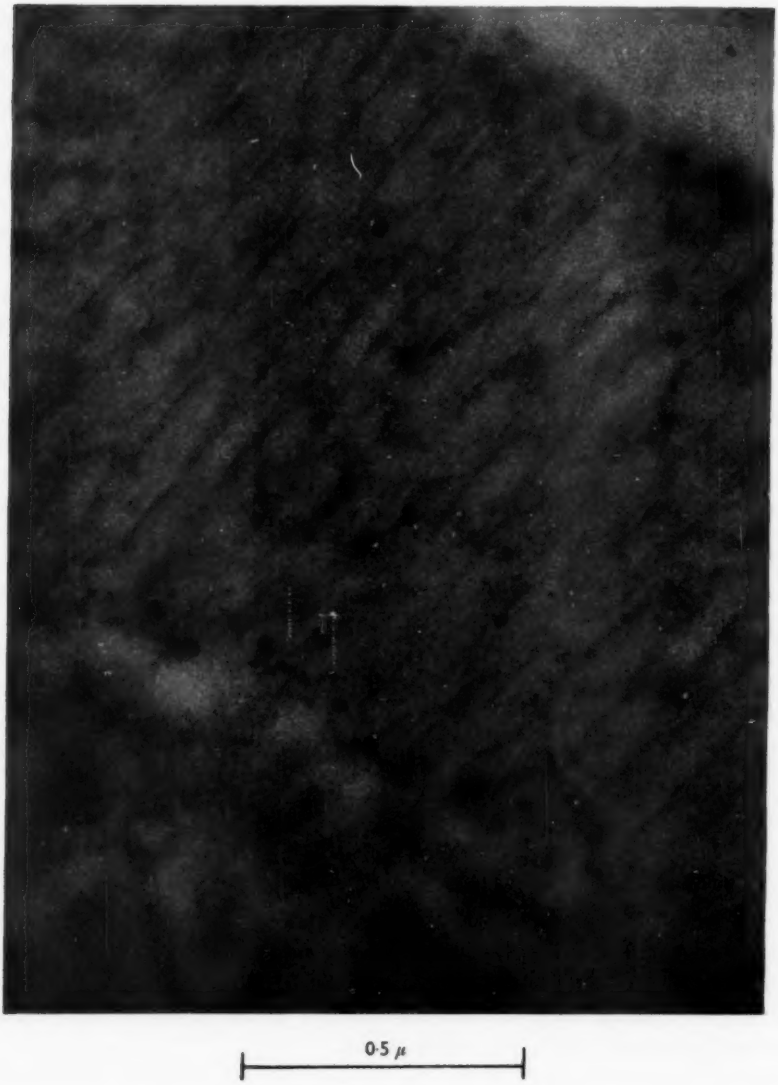

Fig. 1. Electron micrograph showing lipid, represented by small dense bodies, within microvilli.

Fat absorption by the duodenal cells of the rat. By D. Lacy and A. B. Taylor. *Department of Zoology and Comparative Anatomy, St Bartholomew's Medical College, London, E.C.* 1, *and Department of Physiology, University of Illinois, Urbana, U.S.A.*

Rats of one group were starved for 24 hr, and then fed with 0·1–0·4 ml. olive oil and killed at intervals. Rats from a second group received similar treatment except they were not fed. Duodenal cells were examined by electron microscopy (Fig. 1).

In the cells of fat-fed animals, from the microvilli to approximately the upper limit of the Golgi zone, small amounts of a dense substance were observed in the 'space' (25 Å wide) between the two finer membranes of the cell membrane. Dense droplets, about 120–250 Å in diameter and not bounded by a membrane, were seen within microvilli. These droplets were located close to the inner of the two limiting membranes of the microvilli. Similar dense bodies were not seen in the 'core' of the microvilli nor in the terminal web zone. Immediately beneath the terminal web occurred membrane-bound inclusions whose contents ranged from numerous moderately dense droplets each about 100 Å in diameter to a single, markedly dense body about 1000 Å in diameter. Similar inclusions were seen in close proximity to Golgi membranes. Dense bodies, about 700 Å in diameter, were present, frequently in large numbers, in Golgi vacuoles. Bodies of similar density and size also occurred in vacuolar-like spaces between adjacent cells at the level of the Golgi zone and below.

The most likely interpretation of these observations is that they represent the absorption of lipid in a partially or entirely hydrolysed form, and its resynthesis within the membranes which come to enclose it. The lipid accumulates in Golgi vacuoles and is then discharged, probably as neutral fat, into channels (formed by invaginations of the cell membrane) leading to the lateral surface of the cell. These observations do not necessarily preclude the possibility that lipid may be absorbed in a further form and traverse other routes in intestinal epithelial cells.

The localization of an alimentary osmoreceptor in man. By J. N. Hunt. *Physiological Laboratory, Guy's Hospital, London, S.E.* 1

Posterior pituitary hormones of the hippopotamus. By H. Heller and K. Lederis. *Department of Pharmacology, University of Bristol*

Two vasopressins are known to occur in the neurohypophyses of mammals. Ox pituitary glands (Turner, Pierce & du Vigneaud, 1951) and those of man, horse and sheep (Light & du Vigneaud, 1958; Acher, Chauvet &

Lenci, 1958, 1959) contain arginine vasopressin, whereas pig pituitaries contain lysine vasopressin (Popenoe, Lawler & du Vigneaud, 1952). Chromatographic and pharmacological results indicate that arginine vasopressin occurs also in the seven other mammalian species investigated (van Dyke, Adamsons & Engel, 1957; Heller & Lederis, unpublished).

Since, so far, the domestic pig is the only species in which lysine vasopressin has been found, the posterior pituitary hormones of the hippopotamus, which is phyletically related to the Suidae, were studied. Thanks to the co-operation of Professor C. P. Luck, a number of hippopotamus pituitaries were obtained from Uganda. Acetic acid extracts of the glands (which were kept in acetone) were chromatographed on paper according to the procedure of Heller & Lederis (1958), with ox and pig neurohypophysial extracts and purified ox and pig vasopressins for reference. A substance which stimulated the isolated rat uterus was eluted at the R_F of oxytocin and a substance which raised the rat's blood pressure at the R_F of lysine vasopressin. The amount of purified vasopressin at our disposal was too small for amino-acid analysis and further characterization was therefore attempted by pharmacological means. When assayed on the hen's uterus, lysine vasopressin has been found to be approximately 15 times less potent than arginine vasopressin (van Dyke, 1959). In our experiments on hen uteri, pig vasopressin was 9·6 and hippopotamus vasopressin 10·5 times less potent than ox vasopressin. It has also been shown (van Dyke, Engel & Adamsons, 1956) that, relative to its pressor activity, lysine vasopressin has a considerably lower antidiuretic potency in dogs than arginine vasopressin. Dr Mary Pickford was kind enough to test the antidiuretic activity of some of our purified hippopotamus vasopressin in a trained unanaesthetized dog. She found that 3 and 6 milliunits of arginine vasopressin inhibited a water diuresis to a similar degree as either 14 and 28 milliunits of lysine vasopressin or 14·8 and 31·4 milliunits of the hippopotamus hormone.

Thus both pharmacological methods of comparison and the chromatographic findings show that the vasopressor-antidiuretic principle in the posterior pituitary of the hippopotamus is not arginine vasopressin. The results indicate strongly that the neurohypophysis of this species, like that of the domestic pig, contains lysine vasopressin.

The vasopressor:oxytocic ratio in the few hippopotamus neural lobes investigated was consistently below unity, i.e., when assayed in rats, these glands contained less vasopressor than oxytocic activity.

The evolutionary implications of these findings will be mentioned.

REFERENCES

ACHER, R., CHAUVET, J. & LENCI, M. T. (1958). *Bull. Soc. Chim. Biol., Paris*, **40**, 2005.
ACHER, R., CHAUVET, J. & LENCI, M. T. (1959). *C.R. Acad. Sci., Paris*, **248**, 1435.
HELLER, H. & LEDERIS, K. (1958). *Nature, Lond.*, **182**, 1231.
LIGHT, A. & DU VIGNEAUD, V. (1958). *Proc. Soc. exp. Biol., N.Y.*, **98**, 692.
POPENOE, E. A., LAWLER, H. C. & DU VIGNEAUD, V. (1952). *J. Amer. chem. Soc.* **74**, 3713.
TURNER, R. A., PIERCE, J. G. & DU VIGNEAUD, V. (1951). *J. biol. Chem.* **191**, 21.
VAN DYKE, H. B. (1959). *Proc. 21st int. Congr. physiol. Sci. Buenos Aires*, p. 61.
VAN DYKE, H. B., ADAMSONS, K. & ENGEL, S. L. (1957). In *The Neurohypophysis*, ed. HELLER, H. London: Butterworths.
VAN DYKE, H. B., ENGEL, S. L. & ADAMSONS, K. (1956). *Proc. Soc. exp. Biol., N.Y.*, **91**, 484.

The effect of secretin on pancreatic blood flow. By MURIEL JONES. *Physiology Department, St Mary's Hospital Medical School, London, W. 2*

There is no agreement in the literature regarding the effect of secretin on pancreatic blood flow (see Tankel & Hollander, 1957, for references). Some workers have shown an occasional or variable increase and others no change in pancreatic blood flow when the gland is stimulated with secretin. Previous experiments with a photocell method (Holton & Jones, 1960) showed that the blood content of the cat's pancreas increased in response to secretin. Since the blood content and blood flow in an organ do not necessarily change together (Hilton & Holton, 1954) it was of interest to measure blood flow through the pancreas and compare it with blood content. The venous outflow from a portion of the cat's pancreas was measured with a photo-electric drop recorder, the blood being returned to the inferior vena cava.

Simultaneous measurements of blood flow and blood content showed that both increased in response to secretin, with approximately the same time course, the increase preceding the outflow of pancreatic juice. However, in these experiments it was found necessary to use larger doses of secretin to obtain a similar response as the experiment proceeded. Since heparin was used after extensive dissection there was inevitably a slow haemorrhage and it seemed likely that an increase in splanchnic vasomotor tone might account for the diminishing response. To test this hypothesis the blood-flow response to constant doses of secretin was measured when the degree of splanchnic vasomotor tone was deliberately varied. The results show that the blood-flow response was diminished during haemorrhage and during a period of carotid occlusion (when splanchnic vasomotor tone was presumably high) and increased after restoration of the blood volume with a solution of polyvinylpyrrolidone.

The variability in the blood-flow responses in earlier experiments could therefore be due to changes in pancreatic vasomotor tone and this factor might also account for some of the discrepancies reported in the literature.

REFERENCES

HILTON, S. M. & HOLTON, P. (1954). *J. Physiol.* **125**, 138–147.
HOLTON, P. & JONES, M. L. (1960). *J. Physiol.* **150**, 479–488.
TANKEL, H. I. & HOLLANDER, F. (1957). *Gastroenterology*, **32**, 633.

The hormones of the pituitary gland of the dogfish (*Scyliorhinus caniculus*). By J. M. DODD. *Gatty Marine Laboratory, University of St Andrews*

Elasmobranch fish, in common with all vertebrates so far examined, have a full complement of endocrine glands, but the nature and biological properties of their secretions are not completely known.

The pituitary gland of the dogfish is well developed and shows an unusual degree of subdivision. A neurohypophysial system is present, although only one of the two nuclei characteristic of the mammals appears to be represented here, namely the pre-optic nucleus. The neurones which constitute this nucleus communicate with the neurohypophysis via the hypothalamus, and they and their axons can be shown, by the accepted histochemical techniques, to be charged with neuro-secretory material.

The neurohypophysis is a diffuse structure containing a few pituicytes and the endings of the neurosecretory axons. It forms a thin investment over a spheroidal mass of pars intermedia tissue, with which it interdigitates to form a compact neuro-intermediate lobe. The adenohypophysis consists of a tongue-like rostral lobe and an almost separate ventral lobe which is embedded in the chondrocranium.

We have recently confirmed the presence of an oxytocin-like substance in the neuro-intermediate lobe which qualitatively has the properties of mammalian oxytocin though there are significant quantitative differences between the two substances. As a result of a long series of assays for anti-diuretic hormone (ADH) on the conscious rat, we have concluded that there is no separate ADH hormone in the elasmobranch pituitary, the consistently low level of ADH activity contained in acetone-dried powders of dogfish neuro-intermediate lobe being due to the intrinsic ADH activity of the oxytocin (Perks, Dodd & Dodd, 1960).

It has long been known that the neuro-intermediate lobe of the elasmobranch pituitary contains melanophore-expanding hormone, and a good deal is known about the physiology of colour change in these animals.

So far as concerns gonadotrophins, we have demonstrated that both testes and ovary show characteristic and marked signs of degeneration when the ventral lobe of the pituitary is removed, though the gonads remain intact and functional in the complete absence of rostral and neuro-intermediate lobes. Replacement therapy has not yet been attempted and it is not known whether more than one gonadotrophin is present.

The degree to which the thyroid gland is controlled by the pituitary appears slight, though we have some evidence from implantation experiments, ventral lobectomy, and from histochemical studies on the ventral lobe of a fish with a greatly enlarged thyroid gland, that thyroid stimulating hormone (TSH) is present and that it is also located in the ventral lobe. Moreover, in studies on hormone specificity we have shown (Dent & Dodd, unpublished) that the thyroid gland of the hatching stage of the dogfish is highly responsive to TSH from mammalian sources.

REFERENCE

PERKS, A. M., DODD, M. H. I. & DODD, J. M. (1960). *Nature, Lond.* (in the Press).

Ketonaemia and metabolism in the starving rat. By P. A. MAYES
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Gammeltoft (1949) reported that the ketonaemia of the starving rat rises and remains elevated until death. However, other work (Mayes, 1953; Engel, 1957) has shown that ketonaemia rises to a maximum after 48 hr and then subsides to almost normal levels after 6–7 days. This work has now been confirmed and extended to include the concurrent quantitative changes that occur in the liver glycogen, liver fat, blood sugar, abdominal depot fat and urinary nitrogen.

Female rats (200 g approx.) were starved from 1 to 7 days and killed to obtain samples for the above analyses. The methods used have been described previously (Mayes, 1959). A few animals died within the experimental period and in each of these cases the ketonaemia had returned to the normal level before death, and the depot fat was found to be exhausted.

The blood sugar level, which paralleled the concentration of liver glycogen, dropped initially, rose gradually to a maximum when the animals had lost 25–35 % in body weight, and fell precipitously before death. Statistical analysis showed that the inverse relation between the blood sugar level and the ketonaemia was just significant ($P = 0{\cdot}01$–$0{\cdot}05$) over the restricted range of 10–30 % drop in weight. On the other hand, there was a highly significant positive correlation ($P < 0{\cdot}001$), between the limits 10–35 % fall in body weight, of the ketonaemia with both the

concentration of fat in the liver and the quantity of fat present in the abdominal depots. MacKay, Carne, Wick & Visscher (1941) did not consider the degree of ketosis in the starving rat to be significantly related to the liver fat content.

The rate of urinary nitrogen excretion showed an initial fall, and then became constant, indicating that the rise in blood sugar and liver glycogen concentration was not due to any increase in the rate of gluconeogenesis from protein during the course of starvation. A 'pre-mortal rise' occurred after 30 % of the body weight had been lost.

Theoretically the quantity of fat in the liver should be a factor limiting ketogenesis, since it is held to be the chief source of ketone bodies. Since the depots supply fat to the liver during starvation, the quantity of depot fat should also be a limiting factor. The foregoing results indicate that these factors are the principal ones affecting the course of ketonaemia in the starving rat.

REFERENCES

ENGEL, F. L. (1957). *Arch. intern. Med.* **100**, 18.
GAMMELTOFT, A. (1949). *Acta physiol. scand.* **19**, 280.
MACKAY, E. M., CARNE, H. O., WICK, A. N. & VISSCHER, F. E. (1941). *J. biol. Chem.* **141**, 889.
MAYES, P. A. (1953). Ph.D. Thesis: University of London.
MAYES, P. A. (1959). *Biochem. J.* **71**, 459.

J. Physiol. (1960), **152**, *pp.* 1–13
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INFLUENCE OF GESTATIONAL AGE AND HORMONES ON EXPERIMENTAL FOETAL BRADYCARDIA

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A single intravenous injection of adrenaline or noradrenaline into the maternal circulation causes a transient bradycardia of the exposed foetus in the near-term rabbit and guinea-pig (Dornhorst & Young, 1952); this was shown to be due to asphyxia consequent upon the reduction in maternal placental blood flow caused by constriction of the uterine arteries, and not due to any accompanying activity of the uterine muscle. The purpose of the present investigation was to study the influence of gestational age on this asphyxial bradycardia, in the guinea-pig foetus *in utero*. It was found that vasoconstrictor drugs in the maternal circulation caused slowing of the foetal heart at the end of gestation only; the experimental evidence suggests that this is not due to a greater sensitivity of the foetal heart to asphyxia, but to a hormonal sensitization of the maternal placental vessels to vasoconstrictor substances. The possible clinical significance of the findings is discussed.

METHODS

The observations were made on 75 guinea-pigs at gestational ages ranging from the fortieth day of pregnancy to term (67 days). The approximate age of the foetuses was determined before the experiment by palpation and afterwards the crown–rump lengths were measured; foetal ages will be described in these terms, and when reference is made to an approximate age this has been estimated from the values for crown–rump length given by Draper (1920) and Bell (1941).

Light anesthesia was induced and maintained with pentobarbitone sodium (Nembutal, Abbott Laboratories) 20–30 mg/kg, given into the brachial vein under local procaine injection. The maternal arterial pressure was recorded from a saline-filled polythene catheter (No. 3) in the carotid artery. The maternal abdominal wall was opened by a mid-line incision, under saline at 38° C, and the exposed uterus supported on a gauze sling. Intra-amniotic pressure was measured from a saline-filled polythene catheter (No. 2) inserted through the intact uterine wall and membranes. Both pressures were measured with New Electronic Products inductance type manometers and the recordings, together with the foetal e.c.g., were made with the N.E.P. recorder through pencil-type mirror galvanometers with an ultra-violet light source and print-out recording paper.

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